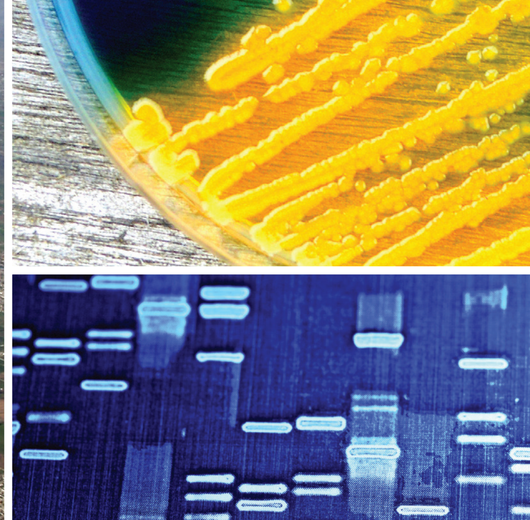
ENVIRONMENTAL MICROBIOLOGY

From Genomes to Biogeochemistry

SECOND EDITION



Eugene L. Madsen

WILEY Blackwell

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Ithaca, New York

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Preface

Over the past 30 years, environmental microbiology has emerged from a rather obscure, applied niche within microbiology to become a prominent, ground-breaking area of biology. Environmental microbiology's rise in scholarly stature cannot be simply explained, but one factor was certainly pivotal in bringing environmental microbiology into the ranks of other key biological disciplines. That factor was molecular techniques. Thanks largely to Dr. Norman Pace (in conjunction with his many students) and Gary Olson and Carl Woese, nucleic acid analysis procedures began to flow into environmental microbiology in the mid-1980s. Subsequently, a long series of discoveries have flooded out of environmental microbiology. This two-way flow is constantly accelerating and the discoveries increasingly strengthen the links between environmental microbiology and core areas of biology that include evolution, taxonomy, physiology, genetics, environment, genomics, and ecology.

This textbook has grown from more than a decade of efforts aimed at presenting environmental microbiology as a coherent discipline to both undergraduate and graduate students at Cornell University. The undergraduate course was initially team-taught by Drs. Martin Alexander and William C. Ghiorse. Later, W.C. Ghiorse and I taught the course. Still later I was the sole instructor. Still later I became instructor of an advanced graduate version of the course. The intended audience for this text is upper-level undergraduates, graduate students, and established scientists seeking to expand their areas of expertise.

Since the first edition of this book in 2008, the discipline of environmental microbiology has made a large number of key advances: in methodologies, in defining evolutionary diversity, and in deepening our understanding of both genetic and biochemical mechanisms of biogeochemical reactions that maintain our world. The goal of his second edition is to make these advances accessible to new students of environmental microbiology. All topics covered by the first edition have been thoroughly updated. A new section has been added to Chapter 5 (diversity) on microbial biogeography. A new section has been added to Chapter 6 (methodologies) that focuses on next-generation sequencing and omics technologies.

Environmental microbiology is inherently multidisciplinary. It provides license to learn many things. Students in university courses will rebel if the

subject they are learning fails to develop into a coherent body of knowledge. Thus, presenting environmental microbiology to students in a classroom setting becomes a challenge. How can so many disparate areas of science (e.g., analytical chemistry, geochemistry, soil science, limnology, public health, environmental engineering, ecology, physiology, biogeochemistry, evolution, molecular biology, genomics, bioinformatics) be presented as a unified body of information?

This textbook is my attempt to answer that question. Perfection is always evasive. However, I have used five core concepts (see Section 1.1) that are reiterated throughout the text, as criteria for selecting and organizing the contents of this book.

The majority of figures presented in this book appear as they were prepared by their original authors in their original sources. This approach is designed to illustrate for the reader that advancements in environmental microbiology are a community effort.

A website with downloadable artwork and answers to study questions is available to instructors at www.wiley.com/go/madsen/enviromicrobio2e.

I hope this book will stimulate new inquiries into what I feel is one of the most fascinating current areas of science. I welcome comments, suggestions, and feedback from readers. I thank the many individuals who provided both direct and indirect sources of information and inspiration. I am particularly grateful to P.D. Butler for her astonishing dedication, kindness, and organizational skills that made completion of the book manuscript possible and to W.C. Ghiorse for his unbounded enthusiasm for the art and science of microbiology and Ian Hewson for reviewing section 6.10. I also apologize for inadvertently failing to include and/or acknowledge scientific contributions from fellow environmental microbiologist friends and colleagues across the globe. (Without imposed boundaries, this writing project would never have been completed.) Forward we go!

Eugene Madsen
Ithaca, New York

About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/madsen/enviromicrobio2e

The website includes:

- An instructors' guide to grading study questions
- Powerpoints of all figures from the book for downloading
- PDFs of all tables from the book for downloading

1

Significance, History, and Challenges of Environmental Microbiology

This chapter is designed to instill in the reader a sense of the goals, scope, and excitement that permeate the discipline of environmental microbiology. We begin with five core concepts that unify the field. These are strengthened and expanded throughout the book. Next, an overview of the significance of environmental microbiology is presented, followed by a synopsis of key scholarly events contributing to environmental microbiology's rich heritage. The chapter closes by reminding the reader of the complexity of Earth's biogeochemical systems and that strategies integrating information from many scientific disciplines can improve our understanding of biosphere function.

Chapter 1 Outline

1.1 Core concepts can unify environmental microbiology
1.2 Synopsis of the significance of environmental microbiology
1.3 A brief history of environmental microbiology
1.4 Complexity of our world
1.5 Many disciplines and their integration

1.1 CORE CONCEPTS CAN UNIFY ENVIRONMENTAL MICROBIOLOGY

Environmental microbiology is inherently multidisciplinary. Its many disparate areas of science need to be presented coherently. To work toward that synthesis, this text uses five recurrent core concepts to bind and organize facts and ideas.

Core concept 1. Environmental microbiology is like a child's picture of a house – it has (at least) five sides (a floor, two vertical sides, and two sloping roof pieces). The floor is evolution. The walls are thermodynamics and habitat diversity. The roof pieces are ecology and physiology. To learn environmental microbiology we must master and unite all sides of the house.

Core concept 2. The prime directive for microbial life is survival, maintenance, generation of adenosine triphosphate (ATP), and sporadic growth (generation of new cells). To predict and understand microbial processes in real-world waters, soils, sediments, and other habitats, it is helpful to keep the prime directive in mind.

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Companion Website: www.wiley.com/go/madsen/enviromicrobio2e

Core concept 3. There is a mechanistic series of linkages between our planet's habitat diversity and what is recorded in the genomes of microorganisms found in the world today. Diversity in habitats is synonymous with diversity in selective pressures and resources. When operated upon by forces of evolution, the result is molecular, metabolic, and physiological diversity found in extant microorganisms and recorded in their genomes.

Core concept 4. Advancements in environmental microbiology depend upon convergent lines of independent evidence using many measurement procedures. These include microscopy, biomarkers, model cultivated microorganisms, molecular biology, and genomic techniques applied to laboratory- and field-based investigations.

Core concept 5. Environmental microbiology is a dynamic, methods-limited discipline. Each methodology used by environmental microbiologists has its own set of strengths, weaknesses, and potential artifacts. As new methodologies deliver new types of information to environmental microbiology, practitioners need a sound foundation that affords interpretation of the meaning and place of the incoming discoveries.

1.2 SYNOPSIS OF THE SIGNIFICANCE OF ENVIRONMENTAL MICROBIOLOGY

With the formation of planet Earth 4.6×10^9 years ago, an uncharted series of physical, chemical, biochemical, and (later) biological events began to unfold. Many of these events were slow or random or improbable. Regardless of the precise details of how life developed on Earth (see Sections 2.3 to 2.7), it is now clear that for ~70% of life's history, prokaryotes were the sole or dominant life forms. Prokaryotes (*Bacteria* and *Archaea*) were (and remain) not just witnesses of geologic, atmospheric, geochemical, and climatic changes that have occurred over the eons; prokaryotes are also active participants and causative agents of many geochemical reactions found in the geologic record. Admittedly, modern eukaryotes (especially land plants) have been major biogeochemical and ecological players on planet Earth during the most recent 1.4×10^9 years. Nonetheless, today, as always, prokaryotes remain the "hosts" of the planet. Prokaryotes comprise ~60% of the total biomass (Whitman et al., 1998; see Chapter 4), account for as much as 60% of total respiration of some terrestrial habitats (Velvis, 1997; Hanson et al., 2000), contribute to one half of global primary production via photosynthesis in marine habitats (Azam and Malfatti, 2007), and also colonize a variety of Earth's habitats devoid of eukaryotic life due to topographic, climatic, and geochemical extremes of elevation, depth, pressure, pH, salinity, heat, or light.

The Earth's habitats present complex gradients of environmental conditions that include variations in temperature, light, pH, pressure, salinity, and both inorganic and organic compounds. The inorganic materials range from elemental sulfur to ammonia, hydrogen gas, and methane and the organic materials range from cellulose to lignin, fats, proteins, lipids, nucleic acids, and

Table 1.1
Microorganisms' unique combination of traits and their broad impact on the biosphere

Traits of microorganisms	Ecological consequences of traits
Small size	Geochemical cycling of elements
Ubiquitous distribution throughout Earth's habitats	Detoxification of organic pollutants
High specific surface areas	Detoxification of inorganic pollutants
Potentially high rate of metabolic activity	Release of essential limiting nutrients from the biomass in one generation to the next
Physiological responsiveness	Maintaining the chemical composition of soil, sediment, water, and atmosphere required by other forms of life
Genetic malleability	
Potential rapid growth rate	
Unrivaled nutritional diversity	
Unrivaled enzymatic diversity	

humic substances (see Chapter 7). Each geochemical setting (e.g., anaerobic peatlands, oceanic hydrothermal vents, soil humus, deep subsurface sediments) features its own set of resources that can be physiologically exploited by microorganisms. The thermodynamically governed interactions between these resources, their settings, microorganisms themselves, and 3.6×10^9 years of evolution are probably the source of metabolic diversity of the microbial world.

Microorganisms are the primary agents of geochemical change. Their unique combination of traits (Table 1.1) cast microorganisms in the role of recycling agents for the biosphere. Enzymes accelerate reaction rates between thermodynamically unstable substances. Perhaps the most ecologically important types of enzymatic reactions are those that catalyze oxidation/reduction reactions between electron donors and electron acceptors. Complex mixtures of electron-rich (donors) and electron-poor (acceptors) occur across Earth's habitats (Chapter 3). Biochemical reactions between these pairs of resources are the basis for much physiological evolution. These biochemical reactions allow microorganisms to generate metabolic energy, survive, and grow. Microorganisms procreate by carrying out complex, genetically regulated sequences of biosynthetic and assimilative intracellular processes. Each daughter cell has essentially the same macromolecular and elemental composition as its parent. Thus, integrated metabolism of all nutrients (e.g., carbon, nitrogen, phosphorus, sulfur, oxygen, hydrogen, etc.) is implicit in microbial growth (Chapters 3 and 7). This growth and survival of microorganisms drives the geochemical cycling of the elements, detoxifies many contaminant organic and inorganic compounds, makes essential nutrients present in the biomass of one generation available to the next, and maintains the conditions required by other inhabitants of the biosphere (Table 1.1). Processes carried out by microorganisms in soils, sediments, oceans, lakes, and groundwaters have a major impact on environmental quality, agriculture, and global climate change. These processes are also the basis for current and emerging biotechnologies with industrial and environmental applications (see Chapter 8). Table 1.2

Table 1.2

Examples of nutrient cycling and physiological processes catalyzed by microorganisms in biosphere habitats. (Reproduced and modified with permission from *Nature Reviews Microbiology* from Madsen, E.L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Rev. Microbiol*. **3**: 439–446. Macmillan Magazines, www.nature.com/reviews)

Nutrient cycle	Process	Nature of process	Typical habitat	References
Carbon	Photosynthesis	Light-driven CO_2 fixation into biomass	Fw, FwS, Ow	Overmann and Garcia-Pichel, 2006; Falkowski, 2012
	Carbon respiration	Oxidation of organic C to CO_2	All habitats	Heemsbergen, 2004; Singh et al., 2010
	Cellulose decomposition	Depolymerization, respiration	Sl	Wilson, 2011
	Methanogenesis	Methane production	FwS, Os, Sw, S1	Hedderich and Whitman, 2006; Schink, 1997; Stams and Plugge, 2009; Schink and Stams, 2013
	Aerobic methane oxidation	Methane becomes CO_2	Fw, Ow, Sl	Smith et al., 2010
	Anaerobic methane oxidation	Methane becomes CO_2	Os, Gw	Boetius et al., 2000; Ettwig et al., 2010; Milucka et al., 2012
Biodegradation	Synthetic organic compounds	Decomposition, CO_2 formation	All habitats	Wackett, 2006; Boxall et al., 2004; Escher and Fenner, 2011; Singh, 2012
	Petroleum hydrocarbons	Decomposition, CO_2 formation	All habitats	Van Hamme et al., 2003; Jeon and Madsen, 2013; Timmis, 2010; Bombach et al., 2010
	Fuel additives (MTBE)	Decomposition, CO_2 formation	Gw, Sl, Sw	Deeb et al., 2003; Hyman, 2013
	Nitroaromatics	Decomposition	Gw, Sl, Sw	Spain et al., 2000; Esteve-Núñez et al., 2001; Ju and Parales, 2010
	Pharmaceuticals, personal care products	Decomposition	Gw, Sl, Sw	Alexander, 1999; Ternes et al., 2004; Ort et al., 2010
	Chlorinated solvents	Compounds are dechlorinated via respiration in anaerobic habitats	Gw, Sl, Sw	Maymo-Gatell et al., 1997; Adrian et al., 2000; Loeffler et al., 2013

Nitrogen	Nitrogen fixation	N_2 gas becomes ammonia	Fw, Ow, Sl	Karl et al., 2002; Martinez-Romero, 2006; Canfield et al., 2010; Martinez-Espinosa et al., 2011; Thamdrup, 2012; Zedr and Kudela, 2012
	Ammonium oxidation	Ammonia becomes nitrite and nitrate	Sl, Sw, Ow, Gw	Ward et al., 2011; Vajrala et al., 2012; Stahl and de la Torre, 2012; Hatzenpichler, 2012; Bock and Wagner, 2006
	Anaerobic ammonium oxidation	Nitrite and ammonia become N_2 gas	Os, Sw, Gw	van Niftrik et al., 2004; Jetten et al., 2009; Harhangi et al,. 2012
	Denitrification	Nitrate is used as an electron acceptor and converted to N_2 gas	Sl, Sw, Os, Fw, Gw	Zumft, 1997; van Breemen et al., 2002; Shapleigh, 2006; Bakken et al., 2012
Sulfur	Sulfur oxidation	Sulfide and sulfur become sulfate	Os, Sw, Gw	Sorokin et al., 2006; Dopson and Johnson, 2012
	Sulfate reduction	Sulfate is used as an electron acceptor and converted to sulfur and sulfide	Os, Gw	Rabus et al., 2006; Barton and Fauqu, 2009
Other elements	Hydrogen oxidation	Hydrogen is oxidized to H^+, electrons reduce other substances	Sl, Os, Sw,	Schink, 1997; Schwartz and Friedrich, 2006; Anantharaman et al., 2013
	Mercury methylation and reduction	Organic mercury is formed and mercury ion is converted to metallic mercury	FwS, Os	Sigel et al., 2005; Barkay et al., 2011
	(Per)chlorate reduction	Oxidants in rocket fuel and other sources are converted to chloride	Gw	Coates and Achenbach, 2004; Ettwig et al., 2012
	Uranium reduction	Uranium oxycation is used as an electron acceptor; hence immobilized	Gw	Lovley, 2003; Williams et al., 2012
	Arsenate reduction	Arsenic oxyanion is used as an electron acceptor; hence toxicity is diminished	FwS, Gw	Oremland and Stolz, 2003; Oremland et al., 2009
	Iron oxidation, acid mine drainage	Iron sulfide ores are oxidized, strong acidity is generated	FwS, Gw	Lovley, 2006; Emerson et al., 2010; Papirio et al., 2013

Fw, freshwater; FwS, freshwater sediment; Gw, groundwater; Os, ocean sediments; Ow, ocean waters; Sl, soil; Sw, sewage.

presents a sampling of the ecological and biogeochemical processes that microorganisms catalyze in aquatic or terrestrial habitats. Additional details of biogeochemical processes and ways to recognize and understand them are presented in Chapters 3 and 7.

1.3 A BRIEF HISTORY OF ENVIRONMENTAL MICROBIOLOGY

Early foundations of microbiology rest with microscopic observations of fungal sporulation (by Robert Hooke in 1665) and "wee animalcules" – true bacterial structures (by Antonie van Leeuwenhoek in 1684). In the latter half of the nineteenth century, Ferdinand Cohn, Louis Pasteur, and Robert Koch were responsible for methodological innovations in aseptic technique and isolation of microorganisms (Madigan et al., 2014). These, in turn, allowed major advances pertinent to spontaneous generation, disease causation, and germ theory.

Environmental microbiology also experienced major advancements in the nineteenth century; these extend through to the present. Environmental microbiology's roots span many continents and countries (Russia, Japan, Europe, and England) and a complex tapestry of contributions has developed. To a large degree, the challenges and discoveries in environmental microbiology have been habitat-specific. Thus, one approach for grasping the history and traditions of environmental microbiology is to recognize subdisciplines such as marine microbiology, soil microbiology, rumen microbiology, sediment microbiology, geomicrobiology, and subsurface microbiology. In addition, the contributions from various centers of training can also sometimes be easily discerned. These necessarily revolved around various investigators and the institutions where they were based.

As early as 1838 in Germany, C.G. Ehrenberg was developing theories about the influence of the bacterium, *Gallionella ferruginea*, on the generation of iron deposits in bogs (Ehrlich et al., 2015). Furthermore, early forays into marine microbiology by A. Certes (in 1882), H.L. Russell, P. Regnard, B. Fischer, and P. and G.C. Frankland allowed the completion of preliminary surveys of microorganisms from far-ranging oceanic waters and sediments (Litchfield, 1976).

At the University of Delft (the Netherlands) near the end of the nineteenth century, M.W. Beijerinck (Figure 1.1) founded the Delft School traditions of elective enrichment techniques (see Section 6.2) that allowed Beijerinck's crucial discoveries including microbiological transformations of nitrogen and carbon, and also other elements such as manganese (van Niel, 1967; Atlas and Bartha, 1998; Madigan *et al*., 2014). The helm of the Delft School changed hands from Beijerinck to A.J. Kluyver, and the traditions have been continued in the Netherlands, Germany, and other parts of Europe through to the present. After training in Delft with Beijerinck and Kluyver, C.B. van Niel was asked by L.G.M. Baas Becking to establish a research program at Stanford University's Hopkins Marine Station (done in 1929), where R.Y. Stainer, R. Hungate, M. Doudoroff, and many others were

trained, later establishing their own research programs at other institutions in the United States (van Niel, 1967).

S. Winogradsky (Figure 1.2) is regarded by many as the founder of soil microbiology (Atlas and Bartha, 1998; Ackert, 2013). Working in the latter part of the nineteenth and early decades of the twentieth centuries, Winogradsky's career contributed immensely to our knowledge of soil and environmental microbiology, especially regarding microbial metabolism of sulfur, iron, nitrogen, and manganese. In 1949, much of Winogradsky's work was published as a major treatise entitled, *Microbiologie du Sol, Problémes et Methods: Cinquante Ans de Recherches. Oeuvres Complétes* (Winogradsky, 1949).

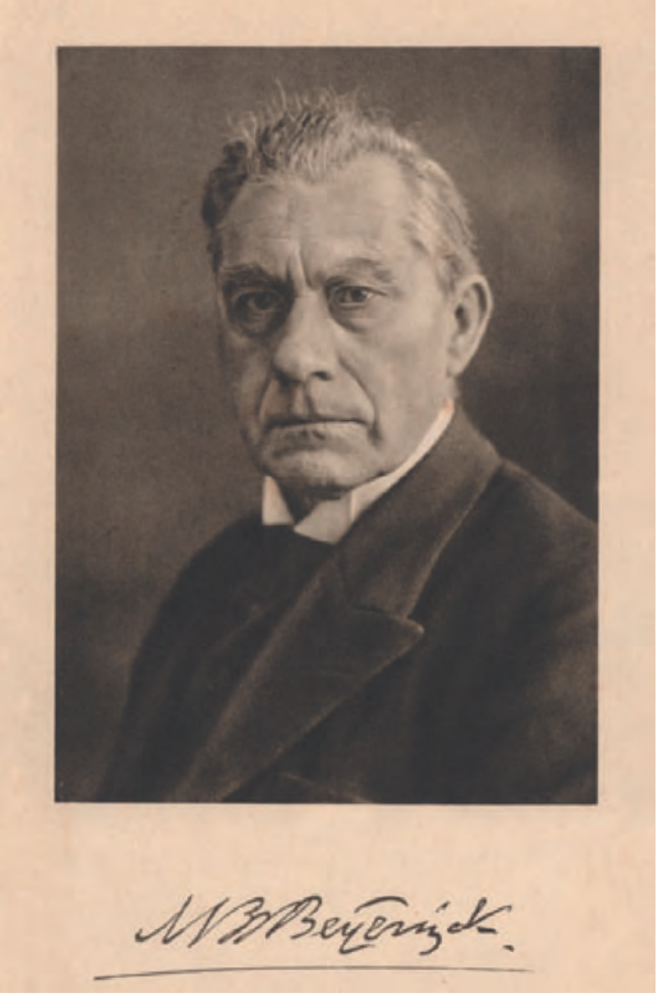

Figure 1.1 Martinus Beijerinck (1851–1931). Founder of the Delft School of Microbiology, M. Beijerinck worked until the age of 70 at the University of Delft, the Netherlands. He made major discoveries in elective enrichment techniques and used them to advance the understanding of how microorganisms transform nitrogen, sulfur, and other elements. (Reproduced with permission from the American Society for Microbiology Archives, USA.)

Many of the marine microbiologists in the early twentieth century focused their attention on photoluminescent bacteria (E. Pluger, E.W. Harvey, H. Molisch, W. Beneche, G.H. Drew, and J.W. Hastings). Later, transformations by marine microorganisms of carbon and nitrogen were explored, as well as adaptation to low-temperature habitats (S.A. Waksman, C.E. ZoBell, S.J. Niskin, O. Holm-Hansen, and N.V. and V.S. Butkevich). The mid-twentieth century marine studies continued exploration of the physiological and structural responses of microorganisms to salt, low temperature, and pressure (J.M. Shewan, H.W. Jannasch, R.Y. Morita, R.R. Colwell, E. Wada, A. Hattori, and N. Taga). Also, studies of nutrient uptake (J.E. Hobbie) and food chains constituting the "microbial loop" were conducted (L.R. Pomeroy).

At Rutgers University, Selman A. Waksman was perhaps the foremost American scholar in the discipline of soil microbiology. Many of the Rutgers traditions in soil microbiology were initiated by J. Lipman, Waksman's predecessor (R. Bartha, personal communication; Waksman, 1952). Waksman produced numerous treatises that summarized the history, status, and frontiers of soil microbiology, often in collaboration with R. Starkey. Among the

Figure 1.2 Sergei Winogradsky (1856–1953). A major contributor to knowledge of soil microbiology, S. Winogradsky described microbial cycling of sulfur and nitrogen compounds. He developed the "Winogradsky column" for growing diverse physiological types of aerobic and anaerobic, heterotrophic and photosynthetic bacteria across gradients of oxygen, sulfur, and light. (Reproduced with permission from the Smith College Archives, Smith College, USA.)

prominent works published by Waksman are "Soil microbiology in 1924: an attempt at an analysis and a synthesis" (Waksman, 1925), *Principles of Soil Microbiology* (Waksman, 1927), "Soil microbiology as a field of science" (Waksman, 1945), and *Soil Microbiology* (Waksman, 1952). A steady flow of Rutgers-based contributions to environmental microbiology continue to be published (e.g., Young and Cerniglia, 1995; Haggblom and Bossert, 2003).

In the 1920s and 1930s, at the University of Wisconsin, E.B. Fred and collaborators, I.L. Baldwin and E. McCoy, comprised a unique cluster of investigators whose interests focused on the *Rhizobium*–legume symbiosis. Several decades later, also at the University of Wisconsin, T.D. Brock and his students made important contributions to microbial ecology, thermophily, and general microbiology. Another graduate of the University of Wisconsin, H.L. Ehrlich earned a Ph.D. in 1951 and, after moving to Rensselaer Polytechnic Institute, carried out studies on the bacteriology of manganese nodules, among other topics. Author of six comprehensive editions of *Geomicrobiology*, H.L. Ehrlich is, for many, the founder of this discipline.

Another University of Wisconsin graduate, M. Alexander, moved to Cornell University in 1955. For four decades prior to Alexander's arrival, soil microbiological research was conducted at Cornell by J.K. Wilson and F. Broadbent. From 1955 to ~2000 Alexander's contributions to soil microbiology examined a broad diversity of phenomena, which included various transformations of nitrogen, predator–prey relations, microbial metabolism of pesticides and environmental pollutants, and advancements in environmental toxicology. Many environmental microbiologists have received training with M. Alexander and become prominent investigators, including J.M. Tiedje.

In Europe (especially in the Netherlands and Germany) Beijerinck's "Delft school" has continued to have a high impact upon the discipline of microbiology, well into the twenty-first century. Key subdisciplines advanced in critically important ways include: taxonomy/systematics (e.g., E. Stackebrandt, K.-H. Schleifler, and W. Ludwig), anaerobic physiology (e.g., R. Thauer, J.G. Kuenen, B. Schink, M.S.M. Jetten, M. Straus, F. Widdel, A. Stams, and W. Zumft), and microbial ecology (e.g., R. Conrad, R. Amann, and G. Muyzer).

Other schools and individuals in Britain, Italy, France, Belgium, and other parts of Europe, Japan, Russia, and other parts of Asia, Africa, Australia, the United States, and other parts of the Americas certainly have contributed in significant ways to advancements in environmental microbiology. An insightful review of the history of soil microbiology, with special emphasis on eastern European and Russian developments, was written by Macura (1974).

The many historical milestones in the development of environmental microbiology (most of which are shared with broader fields of biology and microbiology) have been reviewed by Atlas and Bartha (1998), Brock (1961), Lechevalier and Solotorovsky (1965), Macura (1974), Madigan et al. (2014), van Niel (1967), Waksman (1925, 1927, 1952), Vernadsky et al. (1998), and others. Some of the highlights are listed in Table 1.3.

Table 1.3

Selected landmark events in the history of environmental microbiology

- The first visualization of microscopic life by van Leeuwenhoek in 1684
- The role of microorganisms as causative agents of fermentations discovered by Pasteur in 1857
- The use of gelatin plates for enumeration of soil microorganisms by Koch in 1881
- Nitrogen fixation by nodules on the roots of legumes discovered by Hellriegel and Wilfarth in 1885
- The use of elective enrichment methods, by Beijerinck and Winogradsky, in the isolation of single organisms able to carry out ammonification, nitrification, and both symbiotic and nonsymbiotic nitrogen fixation
- Recognition of the diverse populations in soil (e.g., bacteria, fungi, algae, protozoa, nematodes, insect larvae)
- Documentation of anaerobic cellulose decomposition by Omelianskii in 1902
- The study of sulfur-utilizing phototrophic bacteria by van Niel and others
- The specificity of legume-nodulating bacteria (Fred et al., 1932)
- The discovery and development of antibiotics
- Direct microscopic methods of examining environmental microorganisms via staining and contact-slide procedures
- The development of radiotracer techniques, leading to metabolic activity assays
- A diversity of advancements in analytical chemistry for detecting and quantifying biochemically and environmentally relevant compounds
- Developments in molecular phylogeny (Woese, 1987, 1992; Pace, 1997, 2009)
- The application of molecular methods to environmental microbiology (Olsen et al., 1986; Pace et al., 1986; Amann et al., 1991, 1995; Ward et al., 1993; White, 1994; van Elsas et al., 1997; de Bruijn, 2011a, 2011b; Liu and Jansson, 2010)

As this historical treatment reaches into the twenty-first century, the branches and traditions in environmental microbiology become so complex that patterns of individual contributions become difficult to discern. A complete list of schools, individual investigators, and their respective discoveries is beyond the scope of this section. The author apologizes for his biases, limited education, and any and all inadvertent omissions that readers may notice in this brief historical overview.

1.4 COMPLEXITY OF OUR WORLD

Although we humans are capable of developing ideas or concepts or models that partially describe the biosphere we live in, real-world complexity of ecological systems and subsystems remains generally beyond full scientific description. Figures 1.3 and 1.4 are designed to begin to develop for the reader a sense of the complexity of real-world ecosystems – in this case a temperate forested watershed. The watershed depicted in Figure 1.3 is open (energy and materials flow through it) and features dynamic changes in time and space. The watershed system contains many components ranging from the site geology and soils to both large and small creatures, including microorganisms. Climate-related influences are major variables that, in turn, cause variations in how the creatures and their habitat interact. Biogeochemical processes are manifestations of such interactions. These processes include chemical and physical reactions, as well as the diverse physiological reactions and behavior (Table 1.4). The physical, chemical, nutritional, and ecological conditions for watershed inhabitants vary from the scale of micrometers to kilometers. Regarding temporal variability, in situ processes that

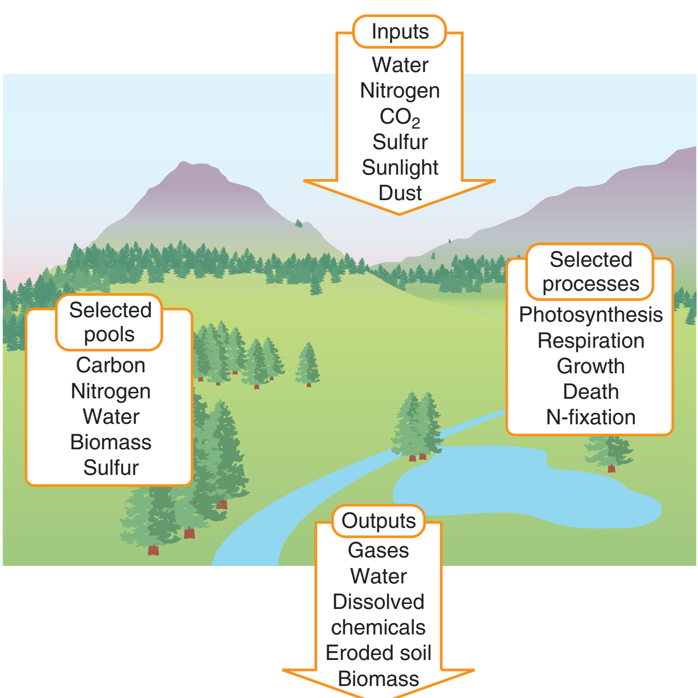

Figure 1.3 Watershed in a temperate forest ecosystem. Arrows show the inputs and outflows for the system. Reservoirs for carbon, nitrogen, and other nutrients include biomass, soil litter layer, soil mineral layer, subsoil, snow, streams, and lakes. Dominant physiological processes carried out by biota include photosynthesis, grazing, decomposition, respiration, nitrogen fixation, ammonification, and nitrification. Key abiotic processes include insolation (sunlight), transport, precipitation, runoff, infiltration, dissolution, and acid/base and oxidation/reduction reactions (see Table 1.4). Net budgets can be constructed for ecosystems; when inputs match outputs, the systems are said to be "steady state".

Table 1.4
Types of biogeochemical processes that typically occur and interact in real-world habitats

Type	Processes
Physical	Insolation (sunlight), atmospheric precipitation, water infiltration, water evaporation, transport, erosion, runoff, dilution, advection, dispersion, volatilization, sorption
Chemical	Dissolution of minerals and organic compounds, precipitation, formation of secondary minerals, photolysis, acid/base reactions, reactions catalyzed by clay-mineral surfaces, reduction, oxidation, organic equilibria, inorganic equilibria
Biological	Growth, death, excretion, differentiation, food webs, grazing, migration, predation, competition, parasitism, symbiosis, decomposition of high molecular weight biopolymers to low molecular weight monomers, respiration, photosynthesis, nitrogen fixation, nitrification, denitrification, ammonification, sulfate reduction, sulfur oxidation, iron oxidation/reduction, manganese oxidation/reduction, anaerobic oxidation of methane, anaerobic oxidation of ammonia, acetogenesis, methanogenesis

directly and indirectly influence fluxes of materials into, out of, and within the system are also dynamic.

At the scale of ~1 m, humans are able to survey habitats and map the occurrence of both abiotic (rocks, soils, gases, water) and biotic (plants, animals) components of the watershed. At this scale, much progress has been made toward understanding ecosystems. Biogeochemical ecosystem ecologists have gained far-reaching insights into how such systems work by performing a variety of measurements in basins whose sealed bedrock foundations allow ecosystem budgets to be constructed (Figure 1.3). When integrated over time and space, the chemical constituents (water, carbon, nitrogen, sulfur, etc.) measured in incoming precipitation, in outflowing waters, and in storage reservoirs (lakes, soil, the biota) can provide a rigorous basis for understanding how watersheds work and how they respond to perturbations (Likens and Bormann, 1995). Understanding watershed (as well as global) biogeochemical cycles relies upon rigorous data sets and well-defined physical and conceptual boundaries. For a given system, regardless of its size, if it is in steady state, the inputs must equal the outputs (Figure 1.3). By the same token, if input and output terms for a given system are not in balance, key biogeochemical parameters of interest may be changing with time. Net loss or gain is dependent on relative rates of consumption and production. Biogeochemical data sets provide a means for answering crucial ecological questions such as: Is the system in steady state? Are carbon and nitrogen accruing or diminishing? Does input of atmospheric pollutants impact ecosystem function? What goods and services do intact watersheds provide in terms of water and soil quality? More details on measuring and modeling biogeochemical cycles are presented in Chapter 7.

Large-scale watershed data capture net changes in complex, open systems. Though profound and insightful, this approach leaves mechanistic microscale cause-and-effect linkages unaddressed. Measures of net change do not address dynamic controls on rates of processes that generate (versus those that consume) components of a given nutrient pool. Indeed, the intricate microscale interactions between biotic and abiotic field processes are often masked in data gathered in large-scale systems. Thus, ecosystem-level biogeochemical data may often fail to satisfy the scientific need for details of the processes of interest. An example of steps toward a mechanistic understanding of the ecosystem process is shown in Figure 1.4. This model shows a partial synthesis of ecosystem processes that govern the fate of nitrogen in a watershed. Inputs, flows, nutrient pools, biological players,

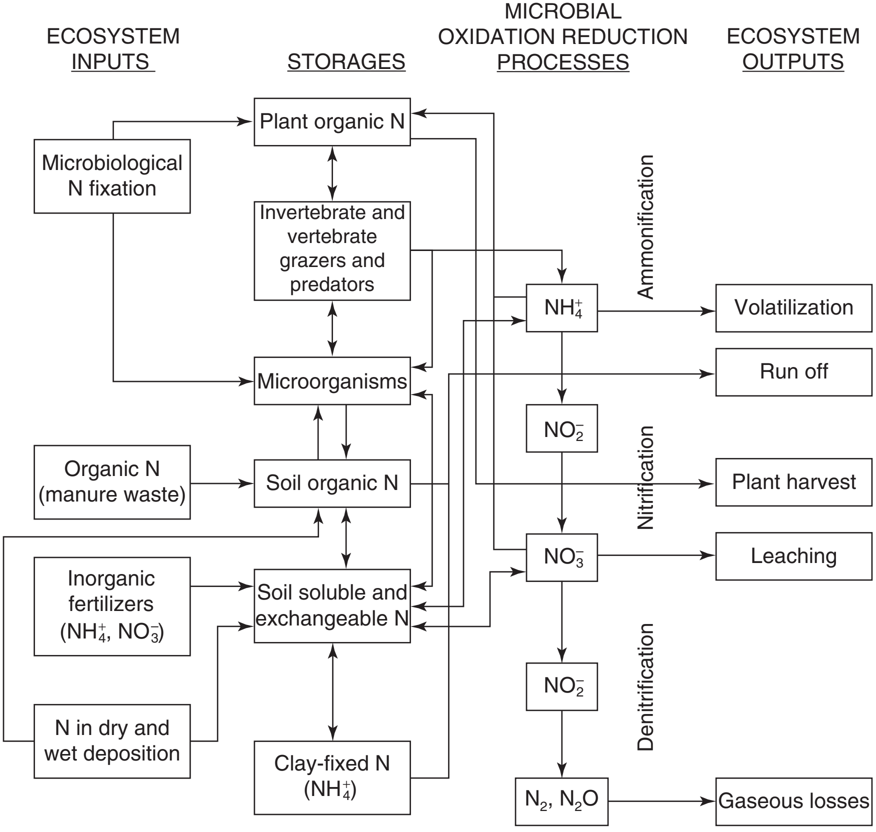

Figure 1.4 Flow model of nitrogen (N) cycling in terrestrial ecosystems. Shown are basic inputs, storages, microbial processes, outputs, and both biotic and abiotic interactions. (Reprinted and modified with permission from Madsen, E.L. 1998. Epistemology of environmental microbiology. *Environ. Sci. Technol.* **32**:429–439. Copyright 1998, American Chemical Society.)

physiological reactions, and transport processes are depicted. Understanding and measuring the sizes of nitrogenous pools, their transformations, rates, fluxes, and the active biotic agents represents a major challenge for both biogeochemists and microbiologists. Yet Figure 1.4 considerably simplifies the processes that actually occur in real-world watersheds because many details are missing and comparably complex reactions and interactions apply simultaneously to other nutrient elements (C, S, P, O, H, etc.). Consider a data set in which concentrations of ammonium (a key form of nitrogen) are found to fluctuate in stream sediments. Interpreting such field measurements is very difficult because the ammonium pool at any given moment is controlled by processes of production (e.g., ammonification or dissimilatory reduction of nitrate to ammonia by microorganisms), consumption (e.g., aerobic and anaerobic ammonia-oxidizing microorganisms, nutrient uptake by plants and many microorganisms), and transport (e.g., entrainment in flowing water, diffusion, dilution, physical disturbance of sediment). Clearly, the many compounded intricacies of nutrient cycling and trophic and biochemical interactions in a field habitat make biogeochemical processes, especially those catalyzed by microorganisms, difficult to decipher.

1.5 MANY DISCIPLINES AND THEIR INTEGRATION

Given the complexity of real-world habitats that are home to microorganisms (see above), what is to be done?

- **How can we contend with complexity?**
- **What approaches can productively yield clear information that enhances our understanding of the role of microorganisms in maintaining our world?**
- **How do microorganisms carry out specific transformations on specific compounds in soils, sediments, and waters?**

Answer: The optimistic answer to these questions is simple. We use the many tools on hand to twenty-first century science.

The principles are sound, the insights are broad, and the sophisticated technologies are ever expanding. To counterbalance the challenges of ecosystem complexity, we can utilize: (i) robust, predictable rules of chemical thermodynamics, geochemical reactions, physiology, and biochemistry; (ii) measurement techniques from analytical chemistry, hydrogeology, physiology, microbiology, molecular biology, omics; and (iii) compound-specific properties such as solubility, volatility, toxicity, and susceptibility to biotic and abiotic reactions. A partial listing of the many areas of science that contribute to advancements in environmental microbiology, with accompanying synopses and references, appears in Table 1.5.

Table 1.5
Disciplines that contribute to environmental microbiology

Discipline	Subject matter and contribution to environmental microbiology	References
Environmental microbiology	The study of microorganisms that inhabit the Earth and their roles in carrying out processes in both natural and human-made systems; emphasis is on interfaces between environmental sciences and microbial diversity	Pepper et al., 2014; Liu and Jansson, 2010; Mitchell and Gu, 2010
Microbial ecology	The study of interrelationships between microorganisms and their biotic and abiotic surroundings	Kirchman, 2008, 2012; Ogilvie and Hirsch, 2012; de Bruijn, 2011a, 2011b; McArthur, 2006
Soil microbiology	Environmental microbiology and microbial ecology of the soil habitat; with emphasis on nutrient cycling, plant and animal life, and terrestrial ecosystems	Paul, 2007; Varma and Oelmüller, 2007
Aquatic microbiology	Environmental microbiology and microbial ecology of aquatic habitats (oceans, lakes, streams, groundwaters)	Canfield et al., 2005; Kirchman, 2008
Microbiology	Holistic study of the function of microbial cells and their impact on medicine, industry, environment, and technology	Madigan et al., 2014
Microbial physiology	Integrated mechanistic examination of bacterially mediated processes, especially growth and metabolism	White et al., 2012; Lengeler et al., 1999; Ljungdahl et al., 2010; Schmitz et al., 2013
Public Health microbiology	Relationships between microbes, environment, and human disease	Burlage, 2012
Geomicrobiology	Interactions between geological and microbiological processes	Ehrlich et al., 2015; Barton et al., 2010
Microscopy	The use of optics, lenses, microscopes, imaging devices, and image analysis systems to visualize small structures	Mertz, 2010; Morris et al., 2010
Biochemistry	Molecular examination of the structure and function of subcellular processes, especially ATP generation, organelles, biopolymers, enzymes, and membranes	Nelson et al., 2008; Berg, et al., 2012
Biotechnology	The integrated use of biochemistry, molecular biology, genetics, microbiology, plant and animal science, and chemical engineering to achieve industrial goods and services	Glick et al., 2009; Vallero, 2010
Biogeochemisty	Systems approach to the chemical reactions between biological, geological, and atmospheric components of the Earth	Schlesinger, 2005; Fenchel et al., 2012; Vernadsky et al., 1998

Table 1.5 *Continued*

Discipline	Subject matter and contribution to environmental microbiology	References
Microbial genetics	Molecular mechanistic basis of heredity, evolution, mutation in prokaryotes, and their biotechnological application	Snyder et al., 2013
Omics	Umbrella term that encompasses bioinfomatics-based systematic analysis of genes (genomics), proteins (proteomics), mRNA (transcriptomics), metabolites (metabolomics), etc.	Schmidt, 2012; Shah and Gharbia, 2010; Mayer, 2011; Marco, 2010; Kraj and Silberring, 2008
Aquatic and soil chemistry	Fundamental reactions of aqueous inorganic and organic chemistry and their quantification based on thermodynamics, equilibrium, and kinetics	Stumm and Morgan, 1996; Tratnyek et al., 2011; Hites and Raff, 2012; Bleam, 2012
Geochemistry	Chemical basis for rock–water interactions involving thermodynamics, mineral equilibria, and solid-, liquid-, and vapor-phase reactions	Drever, 2005; Albaréde, 2009; Holland and Turekian, 2010
Soil science	Study of the intrinsic properties of soils and examination of physical, chemical, and biotic processes that lead to soil formation; the crucial role of soils in agriculture and ecosystems	Brady and Weil, 2007; Shukla and Varma, 2011; Buol et al., 2011; Huang et al., 2012
Limnology	The study of freshwater ecosystems, especially lakes and streams	Wetzel and Likens, 2010
Hydrogeology	The study of the physical flow and migration of water in geological systems	Brooks et al., 2013; Wilderer, 2011
Analytical chemistry	Methods and technologies for detecting, separating, and identifying molecular structures of organic and inorganic compounds	Harris, 2010; Patnaik, 2010; Hites and Raff, 2012
Civil and environmental engineering	Physical, chemical, hydraulic, and biological principles applied to the quantitative design of water supply, wastewater, and other engineering needs	Rittmann and McCarty, 2001; Mihelcik and Zimmerman, 2010
Ecology	Integration of relationships between the biosphere and its inhabitants, with emphases on evolution, trophic dynamics, and emergent properties	Krebs, 2008; Chapin et al., 2011
Environmental science	Multidisciplinary study of how the Earth functions, with emphasis on human influences on life support systems	Miller and Spoolman, 2012; Chiras, 2010

Conceptually, environmental microbiology resides at the interface between two vigorously expanding disciplines: environmental science and microbial ecology (Figure 1.5). Both disciplines (spheres in Figure 1.5) seek to understand highly complex and underexplored systems. Each discipline currently consists of a significant body of facts and principles (green

inner areas of spheres in Figure 1.5), with expanding zones of research (pink bands). But the chances are high that information awaiting discovery (blue areas) greatly exceeds current knowledge. For example, nearly all current information about prokaryotic microorganisms is based upon measurements performed on about 14,000 isolated species. These cultivated species represent approximately 0.1% (or less) of the total estimated diversity of microorganism in the biosphere (estimates range from ~10^7 to 10^{12}, Yarza et al., 2014; see Sections 5.1 to 5.7). The exciting new discoveries in environmental microbiology emerge by examining how microorganisms interact with their habitats (central downward arrow in Figure 1.5).

Thus, the path toward progress in environmental microbiology involves multidisciplinary approaches, assembling convergent lines of independent

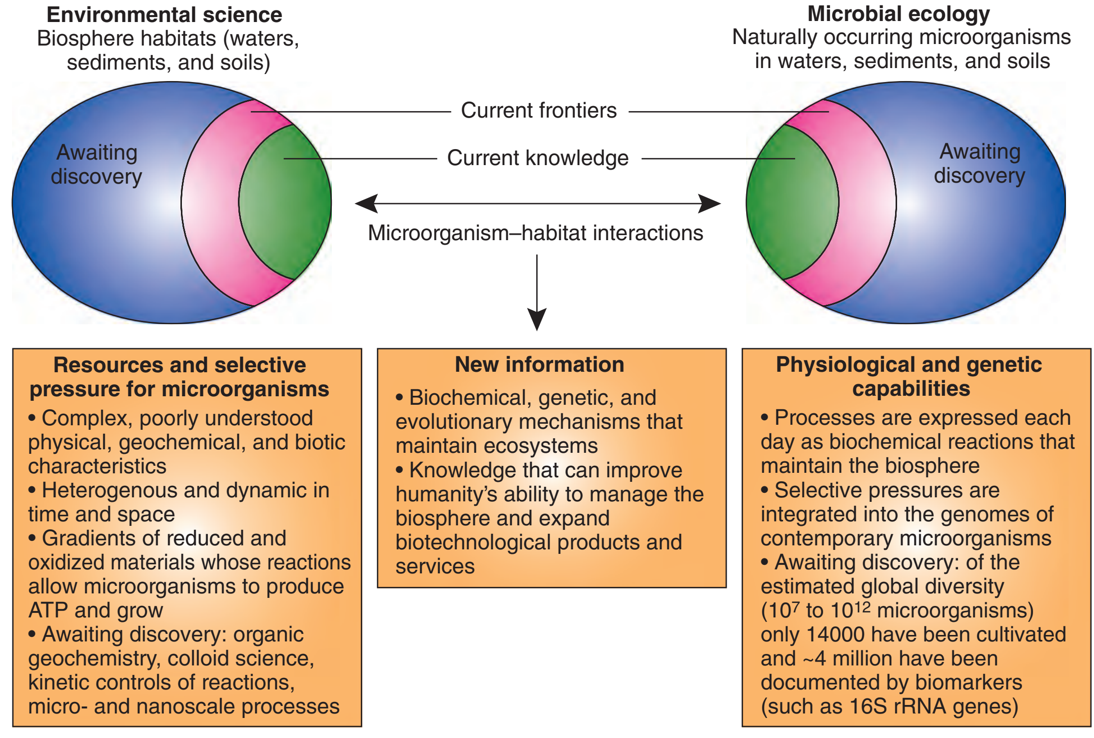

Figure 1.5 Conceptual representation of how the disciplines of environmental science (left sphere) and microbial ecology (right sphere) interact to allow new discoveries at the interface between microorganisms and their habitats. Information in each discipline is depicted as a combination of current knowledge, current frontiers, and knowledge awaiting discovery. Microbial Ecology and Environmental Microbiology have considerable disciplinary overlap (see Table 1.5); nonetheless, advancements in the latter are represented by the central, downward arrow. (Reproduced and modified with permission from *Nature Reviews Microbiology*, from Madsen, E.L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Rev. Microbiol.* **3**:439–446. Macmillan Magazines Ltd, www.nature.com/reviews.)

evidence, and testing alternative hypotheses. Ongoing integration of new methodologies (e.g., from environmental science, microbial ecology, and other disciplines listed in Table 1.5) into environmental microbiology ensures that the number of lines of evidence and the robustness of both their convergence and their tests will increase. A conceptual paradigm that graphically depicts the synergistic relationship between microbiological processes in field sites, reductionistic biological disciplines, and iterative methodological linkages between these disciplines is presented in Figure 1.6.

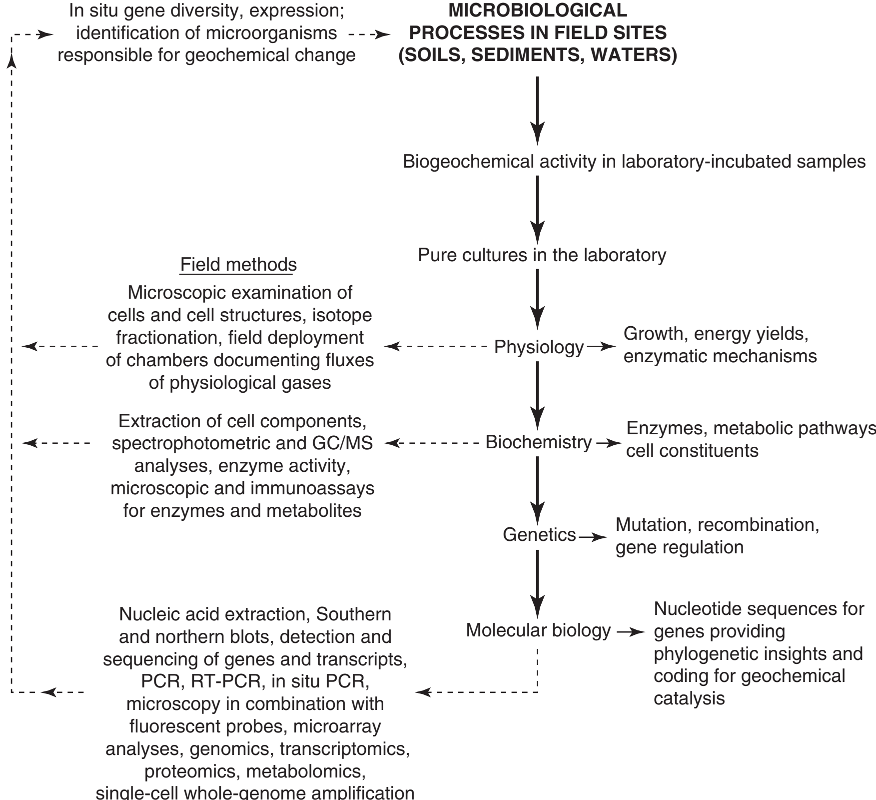

Figure 1.6 Paradigm for how the integration of disciplines and their respective methodologies can extend knowledge of environmental microbiology. Relationships between microorganisms responsible for field biogeochemical processes, reductionistic disciplines, and their application to microorganisms in field sites are depicted. The three different types of arrows indicate sequential refinements in biological disciplines (large downward-pointing solid arrows), resultant information (small arrows pointing to the right), and innovative methodological applications to naturally occurring microbial communities (dashed arrows). GC/MS, gas chromatography/mass spectrometry; PCR, polymerase chain reaction; RT, reverse transcriptase. (Reprinted and modified with permission from Madsen, E.L. 1998. Epistemology of environmental microbiology. *Environ. Sci. Technol*. **32**:429–439. Copyright 1998, American Chemical Society.)

Observations of microorganisms in natural settings instigate a series of procedures progressing through mixed cultures, isolation/cultivation of pure cultures, and physiological, biochemical, genetic, and molecular biological inquiries that each stand alone scientifically. Appreciable new knowledge of naturally occurring microorganisms is gained when advancements from the pure biological sciences are directed back to microorganisms in their field habitats. These methodological advancements (shown as dashed arrows in Figure 1.6; see Chapter 6 for methodologies and their impacts) and the knowledge they generate accrue with each new cycle from field observations to molecular biology and back. Thus, integration of many disciplines is the path forward in environmental microbiology.

STUDY QUESTIONS

1 Core concept 1 presumes a two-dimensional house like that drawn on paper by school children. If you were to expand the concept to three dimensions, then two more walls would be required to keep the "house of environmental microbiology" from falling down. What two disciplines would you add and why? (Hint: for suggestions see Table 1.5.)

2 Core concept 3 uses the phrase "mechanistic series of linkages between our planet's habitat diversity and what is recorded in the genomes of microorganisms found in the world today". This is a hypothesis. If you wanted to test the hypothesis by completing measurements and assembling a data set, what would you do? Specifically, what experimental design would readily test the hypothesis? And what would you measure? What methodological barriers might hamper assembling a useful data set? How might these be overcome? (Hint: Sections 3.2 and 3.3 discusses genomic tools. Answer this question before *and* after reading Chapter 3.)

3 Many names of microorganisms are designed to recognize individual microbiologists who have contributed to the discipline. For instance, the genera *Pasteurella*, *Thauera*, and *Shewanella* are named after people. Similarly, the species designations in *Vibrio harveyii*, *Desulfomonile tiedjei*, *Thermotoga jannaschii*, *Nitrobacter winogradkyi*, and *Acetobacterium woodii* are also named for people. Use the world wide web or a resource like *Bergey's Manual of Systematic Bacteriology* or the *International Journal of Systematic and Evolutionary Microbiology* to discover the legacy of at least one person memorialized in the name of a microorganism.

4 Go for a walk outside to visit a forest, agricultural field, garden, or pond, stream or other body of water. Sit down and examine (literally, and aided by your imagination) the biotic and abiotic components of a cubic meter of water, sediment, or soil. This cubic meter defines a study system. What do you see? Divide a piece of paper into six columns with the headings "Materials and energy entering and leaving", "Inorganic materials", "Organic materials", "Organisms", "Interactions between system components", and "Biological processes". Add at least five entries under each column heading. Then imagine how each entry would change over the course of a year. Compare and contrast what you compiled in your listing with information in Figures 1.3 to 1.6 and Tables 1.2 and 1.4.

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2

Formation of the Biosphere: Key Biogeochemical and Evolutionary Events

This chapter provides an overview of the history of Earth and its forms of life. We review state-of-the-art tools, principles, and logic used to generate information addressing how our world progressed from its ancient prebiotic state to its contemporary biotic state. Key events included: planetary cooling, geochemical reactions at mineral surfaces on the floor of primordial seas, an "RNA world", development of primitive cells, the "last universal common ancestor", anoxygenic photosynthesis, oxygenic photosynthesis, the rise of oxygen in the atmosphere, the development of the ozone shield, and the evolution of higher forms of eukaryotes. The chapter closes by reviewing endosymbiotic theory and key biochemical and structural contrasts between prokaryotic and eukaryotic cells.

Chapter 2 Outline

2.1 Issues and methods in Earth's history and evolution
2.2 Formation of early planet Earth
2.3 Did life reach Earth from Mars?
2.4 Plausible stages in the development of early life
2.5 Mineral surfaces in marine hydrothermal vents: the early iron/sulfur world could have driven biosynthesis
2.6 Encapsulation (a key to cellular life) and an alternative (nonmarine) hypothesis for the habitat of precellular life
2.7 A plausible definition of the tree of life's last universal common ancestor (LUCA)
2.8 The rise of oxygen
2.9 Evidence for oxygen and cellular life in the sedimentary record
2.10 The evolution of oxygenic photosynthesis
2.11 Consequences of oxygenic photosynthesis: molecular oxygen in the atmosphere and large pools of organic carbon
2.12 Eukaryotic evolution: endosymbiotic theory and the blending of traits from *Archaea* and *Bacteria*

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2.1 ISSUES AND METHODS IN EARTH'S HISTORY AND EVOLUTION

- **How do we know what happened long ago?**
- **How old is the Earth?**
- **How did life begin?**
- **When did life begin?**
- **How have life and the Earth changed through the ages?**

These and related questions have likely been pondered by humans for thousands of years. In our quest for understanding extant microorganisms that dwell in biosphere habitats, it is essential to place them in historical, metabolic, and evolutionary context. To achieve this, we would ideally be able to superimpose continuous, independent timelines derived from the geologic record, the fossil record, the climate record, the evolutionary record, and the molecular phylogenetic record. Conceivably this superimposition could allow cause-and-effect interactions to be documented, linking specific events such as changes in atmospheric composition, glaciation, tectonic movements, and the rise and fall of biotic adaptations. This ideal has not yet been achieved at high resolution. Instead, we have only glimpses here and there of our planet's complex, shrouded past (Table 2.1). However, recent advances have made progress toward achieving a synthesis that may solve the puzzles of Earth's history. The key tools used to discover and decipher planetary history are listed in Table 2.2 and further explained in Boxes 2.1 and 2.2. By knowing Earth's global distribution of land forms, rocks, and minerals, geologists have identified where to look for clues about ancient Earth and life's beginnings (Figure 2.1). Discovery of the clues and their assembly into a convincing, coherent body of knowledge is ongoing – reliant upon insights from geology, paleontology, nuclear chemistry, analytical chemistry, experimental biochemistry, as well as molecular phylogeny (Table 2.2).

2.2 FORMATION OF EARLY PLANET EARTH

Explosions from supernovae 4.6×10^9 years ago are thought to have instigated the formation of our solar system (Nisbet and Sleep, 2001; Nisbet and Arndt, 2012). The inner planets (Earth, Mars, Venus, and Mercury) were produced from collisions between planetesimels. Early Earth featured huge pools of surface magma, which cooled rapidly (~2×10^6 years) to ~100 °C. Later, water condensed, creating the oceans. Volcanism and bombardment by meteors were common (Abramov and Mojzsis, 2009). These collisions are thought to have repeatedly heated the oceans to >100 °C, causing extensive vaporizing of water. Our moon was likely to have formed 4.5×10^9 years ago when molten mantle was ejected into orbit after Earth

Table 2.1

Key event and conditions of early Earth

Time (10^9 years before present)	Events	Conditions
4.6	• Colliding planetesimals • Earth formed • Moon formed • Loss of water and hydrogen from atmosphere • Volcanism • Cooling of surface • Faint young Sun • Glaciation?	• Heat • Meteor bombardment and impacts • Lightning • UV radiation • Hot oceans followed each bombardment • Atmosphere: N_2, CO_2, CO, H_2, H_2O, NH_3, CH_4, HCN • Ocean chemistry: H_2S, Fe^{2+}, heavy metals
4.2	• Bombardment ceased (?)	
4.0	• RNA world, iron/sulfur world • Last universal common ancestor • Separation of bacterial and archael cell lineages • Anoxygenic photosynthesis (^{12}C enrichment in geologic record)	• Sulfide-dominated ocean waters and pyrite (FeS_2) deposition
3.5	• Nucleated (eukaryotic) line of descent	
3.5–3.4	• Fossils resembling bacterial filaments and stromatolite microbial mats; microfossils of sulfate-reducing and sulfur-oxidizing bacteria; sulfate reduction (^{32}S enrichment in geologic record)	• Banded iron geological formations
2.7	• Biomarker for cyanobacteria	
2.4	• Biomarker for primitive eukaryotes	• Red bed geologic formations
1.8		• Banded iron formation ceases
1.4	• Signs of oxygen at low concentration in atmosphere	• Oxygen in atmosphere ~1%
0.6	• Nucleated eukaryotic algae • Biomarkers for early animals (sea sponge)	• Ozone shield • Oxygen in atmosphere ~21% • Oxygenation of the deep ocean
0.4	• Cambrian explosion of eukaryotic diversity	
0.1	• Dinosaurs, higher plants, mammals	

was struck by another planet about the size of Mars. Bombardment diminished perhaps by 4.2–4.0 × 10^9 years ago. The scale of geologic time, from planet formation to the present, is shown in Figure 2.2.

The influence of ancient atmospheres upon surface conditions was critical. Abundances of greenhouse and other gases (especially CO_2, NH_3, H_2O, CO, CH_4, HCN, N_2, and H_2) were probably highly dynamic. In combination

Table 2.2
Scientific tools providing information about Earth history and evolution

Discipline	Tool	Insights
Geology	Global surveys of terrestrial and oceanic rocks	Sedimentary, igneous, and metamorphic formations reveal tectonic and other processes governing Earth's evolution
Nuclear chemistry	Radioisotopic dating	Ages of rocks, minerals, and their components are revealed
Paleontology	Fossil record	Organism structures preserved in stratified sediments provide records of evolution
Analytical chemistry of biomarkers	Analytical determination of biomolecules via chromatography and mass spectrometry	Molecular remnants of biomolecules (membranes, pigments, cell walls, etc.) document ancient biota
Analytical chemistry of isotopic ratios	Isotope ratio mass spectrometry	Enzyme reactions favor substrate molecules composed of lighter atoms. Biomass assimilates the lighter isotope and the remaining isotopic pool becomes "heavier" for a given process
Experimental biochemistry	Model systems that simulate ancient Earth	Discovery of precursors of cellular structures and their self-assembling properties
Molecular phylogeny	Sequencing and analysis of informational biomolecules	Alignment of sequences from DNA, proteins, and other molecules allow evolutionary inferences to be drawn, especially regarding the three domains of life
Mineralogy and geochemistry	X-ray diffraction and wet-chemical analysis of rocks	Chemical reactions and reactants of past ages can be inferred from the composition and oxidation/reduction status of ancient sediments
Biochemistry	Comparative biochemistry of cellular materials	Trends in evolutionary relatedness among and between members of *Bacteria*, *Archaea*, and *Eukarya*

with variations in solar radiation, atmospheric conditions may have contributed to periods of high surface temperatures (~100°C) that perhaps alternated with low-temperature (glaciated) periods. It is thought that water was kept from freezing when our Sun was young and faint by greenhouse warming stemming from high concentrations of H_2 and N_2 in the atmosphere (Wordsworth and Pierrehumbert, 2013). Clearly, conditions on prebiotic Earth were turbulent – characterized by fluctuating temperatures, aqueous reactions with magma, input of materials from meteorites (including organic carbon), electrical discharges from the atmosphere, and reduced (nonoxidizing) gases in the atmosphere.

Box 2.1

The age of the Earth and biota

Radioactive decay in rocks

Measurements performed on rock containing radioactive elements (nuclides) can reveal the age of the rock. For example, ^{238}U (half-life = 4.5×10^9 years) decays to helium and ^{206}Pb. Each atom of ^{238}U that decomposes forms eight atoms of helium (with total mass 32), leaving one atom of ^{206}Pb. In 4.5×10^9 years, 1 g of ^{238}Pu becomes 0.5000 g of ^{238}U and 0.174 g of He and 0.326 g of ^{206}Pb. If analyses document nuclides present in the rock in the above ratios, the age would be 4.5×10^9 years. Other radioactive elements have their own characteristic half-lives and decay products; thus, ratios of $^{235}U/^{207}Pb$, $^{232}Th/^{208}Pb$, $^{40}K/^{40}Ar$, and $^{87}Rb/^{87}S$ are also insightful for determining the ages of rocks. The present estimate of the age of Earth and the other inner planets in our solar system is 4.6×10^9 years.

Carbon dating of life

About one in every 10^{12} carbon atoms on Earth is radioactive (^{14}C) and has a half-life of 5760 years. Carbon dioxide, radioactive and nonradioactive alike, is absorbed by plants and incorporated into the biota that consume plants. When a plant or animal dies, its ^{14}C atoms begin undergoing radioactive decay. After 11,520 years (two half-lives), only one-quarter of the original radioactivity is left. Accordingly, by determining the ^{14}C radioactivity of a sample of carbon from wood, flesh, charcoal, skin, horn, or other plant or animal remains, the number of years that have passed since the carbon was removed from atmospheric input of ^{14}C can be determined.

Box 2.2

Biomarkers and isotopic fractionation

Biomarkers

Biomarker compounds are molecules of known biosynthetic origin. As such, their detection in geologic samples (ancient buried soils, rocks, sediments) associates the biosynthetic pathway and/or its host organism with the source material. Biomarker geochemistry has been routinely applied to petroleum exploration and also has been insightful in analyzing rocks (e.g., 2.7×10^9-year-old shales from northwestern Australia; Brocks et al., 1999). Examples of biomarkers that have been extracted from rocks are: 2-methyl-hopanes (derived from 2-methyl-bacteriohopane polyols, which are membrane lipids synthesized by cyanobacteria), pyrrole molecules (essential building blocks of the photosynthetic (chlorophyll) and respiratory (cytochrome) apparatus), and C_{30} sterols (Love et al., 2009), indicative of animal life (marine sponges) 635×10^6 years ago.

Isotopic fractionation

Many chemical elements on Earth occur as mixtures of atoms with differing numbers of neutrons in their nuclei. For instance, the natural abundance of stable (nonradioactive) carbon

Box 2.2 ***Continued***

with six neutrons and six protons (^{12}C) is 98.9%, while ~1.1% of the total carbon pool has seven neutrons (^{13}C). Enzymes involved in photosynthesis show subtle selectivity in acting on their substrate (CO_2) when it is composed of the lighter (^{12}C) carbon isotope. Photosynthesis fixes atmospheric CO_2 into biomass; therefore, the biomass is enriched in ^{12}C – it is "light". Correspondingly, as ^{12}C–CO_2 is removed from the atmosphere, the remaining pool is enriched in ^{13}C–CO_2 – it becomes "heavy". Such shifts in isotopic ratios can be detected in carbon and other elements (especially sulfur; ^{32}S-enriched sulfide minerals indicate sulfate reduction, Table 2.1) extracted from ancient rocks. Because enzymatic selectivity is the only known mechanism for such shifts, these constitute evidence for biological processes.

As listed in Table 2.2, carbon isotopic ratios are determined using an analytical technique known as isotope ratio mass spectrometry. The means of expressing the ratio uses a "del ^{13}C" value, which contrasts the $^{13}C/^{12}C$ ratio in a sample with that of a standard:

$$\delta^{13}C = \frac{(^{13}C/^{12}C\ \text{sample}) - (^{13}C/^{12}C\ \text{standard})}{^{13}C/^{12}C\ \text{standard}} \times 1000$$

Note that the $\delta^{13}C$ value becomes negative ("light") if the sample is depleted in ^{13}C, relative to the standard. Extensive surveys of carbon pools found in nature have been cataloged. This compilation of characteristic values (Grossman, 2002) allows the origin of many carbon pools to be ascertained:

Pool of carbon	Range of $\delta^{13}C$
Marine carbonate	−5 to +8
Atmospheric CO_2	−8 to −5
Calvin cycle plants (C_3)	−27 to −21
C_4 plants	−17 to −9
Petroleum	−32 to −22
Thermogenic methane	−48 to −34
Microbial methane	−90 to −48
Cyanobacteria	−30 to −18
Purple sulfur bacteria	−35 to −20
Green sulfur bacteria	−20 to −9
Recent marine sediments	−35 to −10

2.3 DID LIFE REACH EARTH FROM MARS?

There is a general consensus that stable isotopic ratios (see Tables 2.1, 2.2 and Box 2.2) in graphite isolated from the Isua supracrustal belt (West Greenland; see Figure 2.1) prove that life, manifest as anoxygenic photosynthesis, was present 3.8×10^9 years ago (Nisbet and Sleep, 2001; Nisbet and Arndt, 2012). Before focusing upon a plausible scenario of how life evolved on Earth, an alternative, perhaps equally plausible, hypothesis

must briefly be considered: Panspermia. In the early history of our solar system, Earth, Venus, and Mars, close neighbors, were simultaneously undergoing planetary development. Mars, in particular, featured an abundance of water and other geochemical conditions that may have been favorable for, and led to the development of, life. Meteor bombardment was rampant in the early solar system. Such collisions transferred materials between planets. Microbial life buried within the interstices of Martian rocks may have survived transit to Earth and landed in seas capable of supporting growth. Once seeded, Earth-specific evolutionary forces would have taken hold. The discussion below on the possible origin of life applies to Earth, as well as other planets.

Figure 2.1 Map of the world showing locations of rock formations that provide insights into the coevolution of life and the Earth.

2.4 PLAUSIBLE STAGES IN THE DEVELOPMENT OF EARLY LIFE

Among life's many attributes is the creation of order out of disorder. The Second Law of Thermodynamics mandates that order be created at the expense of energy and the production of entropy. Mechanistically, life is manifest as the synthesis of molecular structures that facilitate metabolic and genetic processes. Such structures are antientropic – requiring energy for synthesis and assembly. Fortunately, abundant physical energy sources prevailed in sterile, prebiotic Earth: these included heat, UV radiation, and electrical discharges (lightning). Investigations by S. Miller in the 1950s proved that amino acids can be chemically synthesized under conditions simulating ancient seas. This de novo synthesis of organic compounds, supplemented with ones borne on meteorites, leaves little doubt that an organic geochemical broth developed.

The transition from a soup of life's primitive potential building blocks to advanced cellular life is thought to have proceeded through many stages of increasing complexity (Figures 2.2 and 2.3). The fundamental conceptual foundation in developing and testing theories about the origin of life is "getting here from there". We need to define "here", define "there", and do our best in devising feasible, continuous connections between the two. "Here" refers to the highly complex characteristics of modern cellular life: heredity (DNA), transcription (RNA), translation (ribosomes), catalysis (proteins), compartmentalization (membrane-enclosed cells and organelles), metabolic

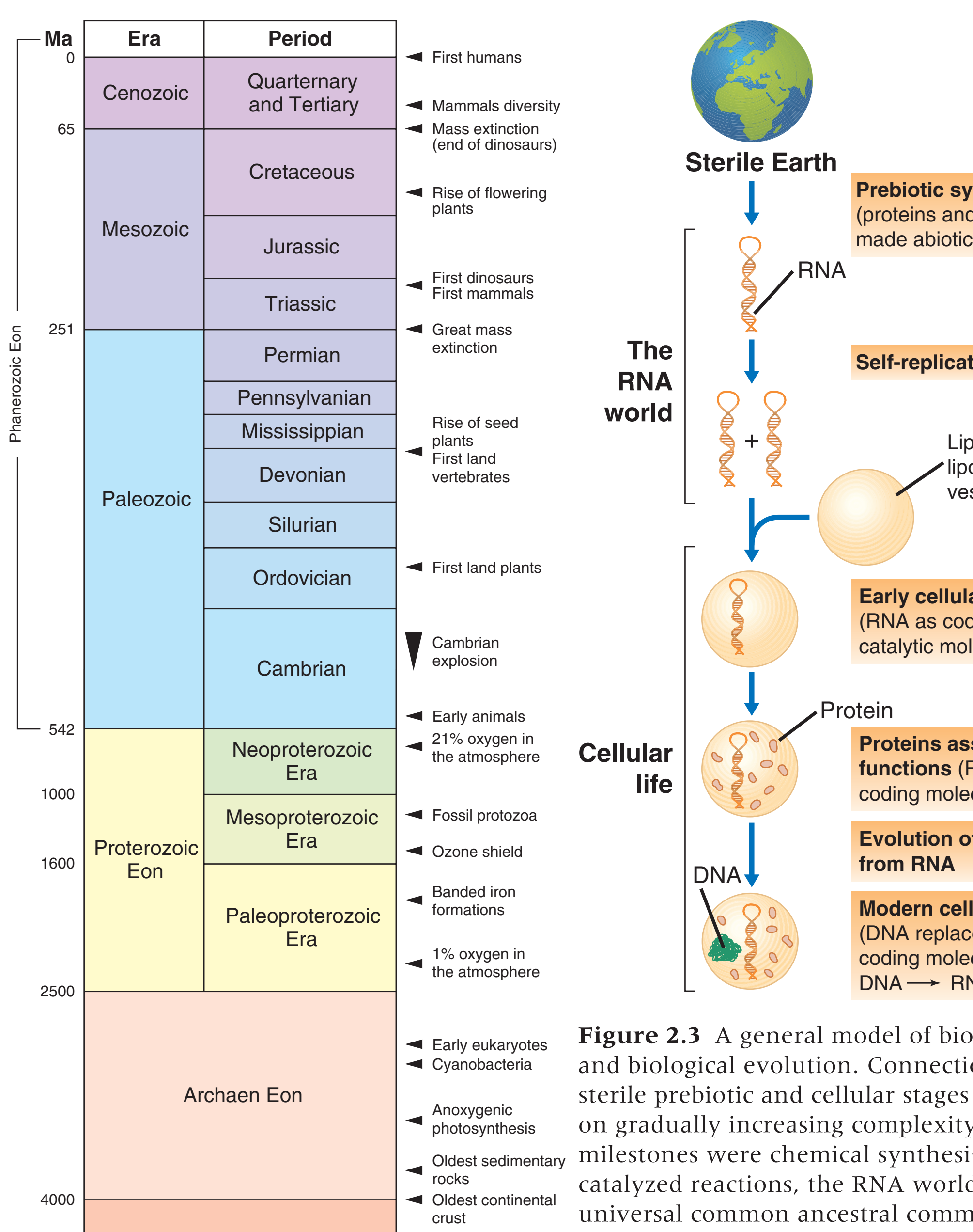


Figure 2.2 Geological timescales and evolutionary events. Note that the scale is not linear (Ma, 10^6 years ago). (Modified from Knoll, A.H. 2003. *Life on a Young Planet*. Copyright 2003, Princeton University Press. Reprinted by permission of Princeton University Press.)

Figure 2.3 A general model of biochemical and biological evolution. Connections between sterile prebiotic and cellular stages of life rely on gradually increasing complexity. Key milestones were chemical synthesis, surface catalyzed reactions, the RNA world, the last universal common ancestral community, and compartmentalization to form free-living cells. (From Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 304. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ.)

energy production (e.g., electron transport, adenosine triphosphate (ATP), and ATP synthase), and biosynthesis (using energy for cellular replication). Figure 2.3 presents a possible scenario of events leading from sterile Earth through the "RNA world" to cellular life. RNA-based proto-life is a likely intermediary step because RNA has both self-replication and catalytic traits. However, proteins are superior to RNA as catalysts and DNA is superior to RNA as a stable reservoir of genetic information. In the scenario shown in Figure 2.3, RNA's role in metabolism gradually shifted to intermediary template between information-bearing DNA and substrate-specific protein catalysts. Several of the key steps thought crucial to life's development are discussed below.

Science and the citizen

War of the Worlds and martian life

Headline news from English literature and American radio

The 1898 novel by H.G. Wells depicted an invasion of England by aliens from Mars. Meteor-like, cylindrical spaceships landed throughout the countryside. Tentacled creatures assembled armed fighting machines that brought fear and destruction to humanity. In 1938, the American writer, director, and actor, Orson Welles broadcast a radio show based on *War of the Worlds*. The radio broadcast was so realistic that it caused widespread panic among radio listeners.

SCIENCE: real martian life?

Contrary to H.G. Wells' and Orson Welles' depiction of sophisticated martians with advanced technology, the real news about extraterrestrial life is microbial. On August 16, 1996, an article by McKay et al., entitled "search for past life on Mars: possible relic biogenic activity in martian meteorite ALH84001" appeared in the prestigious *Science* magazine. The authors' hypothesis was that microorganisms on Mars carried out metabolic activities that caused the formation of carbonate globules, magnetite mineral, iron sulfide mineral, and cell biomass. The latter was proposed to have been converted to polycyclic aromatic hydrocarbons (see Section 8.3 and Box 8.7) during transport from Mars to Earth.

The final paragraph of the article summarized the information presented that argued for past microbial life on Mars.

> In examining the martian meteorite ALH84001 we have found that the following evidence is compatible with the existence of past life on Mars:
> (i) an igneous Mars rock (of unknown geologic context) that was penetrated by a fluid along fractures and pore spaces, which then became the sites of secondary mineral formation and possible biogenic activity;
> (ii) a formation age for the carbonate globules younger than the age of the igneous rock;
> (iii) scanning electron micrograph and transmission electron micrograph images of carbonate globules and features resembling terrestrial microorganisms, terrestrial biogenic carbonate structures, or microfossils;
> (iv) magnetite and iron sulfide particles that could have resulted from oxidation and reduction reactions known to be important in terrestrial microbial systems; and
> (v) the presence of polycyclic aromatic hydrocarbons associated with surfaces rich in carbonate globules.
>
> None of these observations is in itself conclusive for the existence of past life. Although there are alternative explanations for each of these phenomena taken individually, when they are considered collectively, particularly in view of their spatial association, we conclude that they are evidence for primitive life on early Mars.

About 3 years subsequent to McKay and colleague's publication, each of the five arguments for ancient martian microbial life was challenged in the scientific literature (e.g., Anders, 1996; Borg et al., 1999). Alternative, largely chemical, mechanisms were found for the formation of what McKay et al. had argued to be biogenic structures. Now, the general consensus is that this hypothesis about ancient martian life has been disproven.

The intellectual and scientific exercise of seeking extraterrestrial life has, nonetheless, been beneficial to environmental microbiology. A new discipline has been born – astrobiology. Advances in the astrobiology scientific community have enabled it to be far better prepared to document new forms of microbial life.

Research essay assignment

The terms "extremophile", "exobiology", and "astrobiology" have been used extensively in both the scientific and nonscientific literature. After finding about six published works addressing these topics, write a 3–5 page essay that merges two aspects of astrobiology: (i) the human preoccupation with alien life forms and (ii) the genesis and goals of the astrobiology field of science.

2.5 MINERAL SURFACES IN MARINE HYDROTHERMAL VENTS: THE EARLY IRON/SULFUR WORLD COULD HAVE DRIVEN BIOSYNTHESIS

Although some organic chemicals can be synthesized from simple inorganic gases in the presence of electrical discharges (see above), the open waters of ancient seas are not the likely site of life's key early developmental stages. One reason for this is that water participates in hydrolytic cleavage reactions – which are not conducive to building complex organic molecules. In contrast, mineral surfaces (particularly iron monosulfide (FeS) minerals lining microporous rocks at the bottom of ancient seas) are currently thought to be the site where early life began (Martin and Russell, 2003; Martin et al. 2008; Lombard et al., 2012). These rock formations, analogous to today's hydrothermal vents (Figure 2.4), offered three-dimensional compartments of diffusion-limited hydrophobic surfaces that could bind and concentrate organic compounds. In addition, FeS and nickel monosulfide (NiS) catalysts lining the porous cavities are capable of forging carbon–carbon bonds (Huber and Wachtershauser, 2006; Wachtershauser 2006, 2010, 2013;

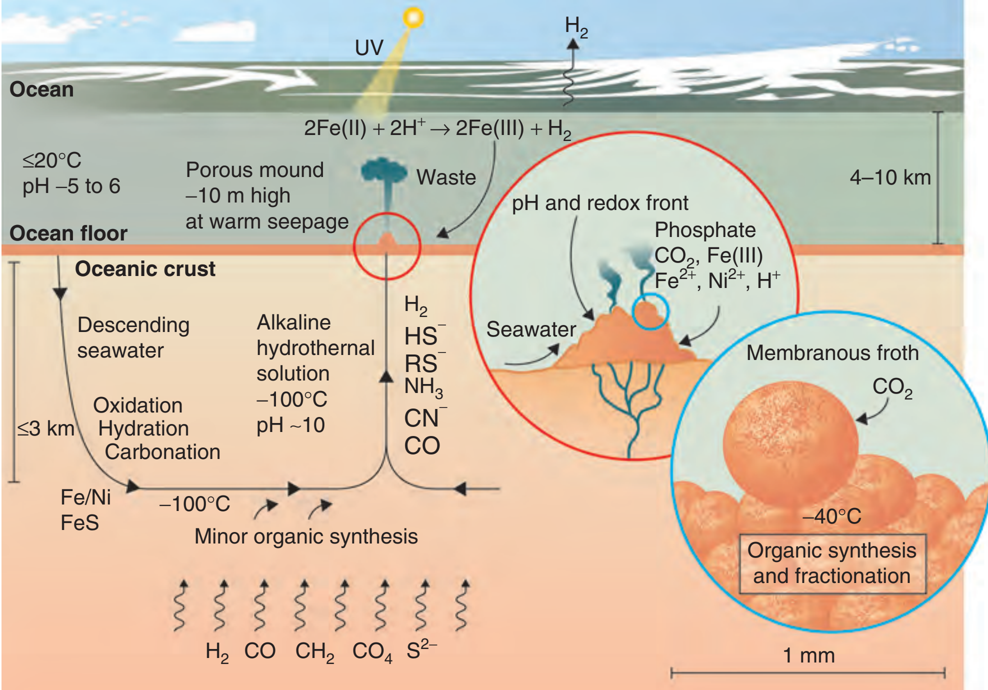


Figure 2.4 The submarine setting for the emergence of life. (From Russell, M.J. 2003. Geochemistry: the importance of being alkaline. *Science* **302**:580–581. Reprinted with permission of AAAS.)

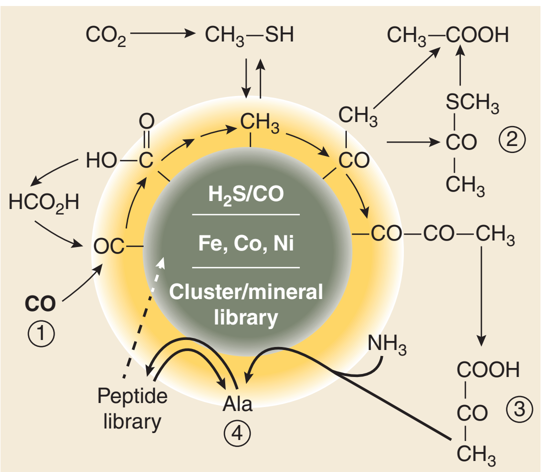


Figure 2.5 Reactions in the iron/sulfur world. Shown are the proposed reaction schemes for primordial molecules at catalytic surfaces in the iron/sulfur world. Reaction steps lead to the conversion of carbon monoxide (1) to a variety of key biomolecules, including: methyl thioacetate (2), pyruvate (3), and alanine (4). (From Wachtershauser, G. 2000. Origin of life: life as we don't know it. *Science* **289**:1307–1308. Reprinted with permission of AAAS.)

see Section 7.1). The geochemical context of these porous mineral surfaces was along gradients of oxidation/reduction potential, and temperature where alkaline, sulfide-rich hydrothermal fluids mixed with somewhat acidic Fe(II)-containing waters of the ocean floor (Lane et al., 2010). Under such conditions oxidative formation of pyrite (FeS_2) occurs spontaneously:

$$FeS + H_2S \rightarrow FeS_2 + H_2 \quad \Delta G = -38.4 \text{ kJ/mol}$$

This type of exothermic reaction produces reducing power (H_2), often essential in biosynthetic reactions. It also can drive the autocatalytic assembly of complex organic molecules (Figure 2.5; Wachtershauser, 1990, 1992, 2006, 2010, 2013). Current thought (Martin and Russell, 2003) holds that the chemistry of the RNA world (including reduction of CO and CO_2; peptide bond formation; synthesis of nucleotides; and formation of thioester precursors of ATP) occurred in these FeS cavities.

2.6 ENCAPSULATION (A KEY TO CELLULAR LIFE) AND AN ALTERNATIVE (NONMARINE) HYPOTHESIS FOR THE HABITAT OF PRECELLULAR LIFE

As defined above, proto-life (organic catalysis and replication) was confined to the rocky pores where the RNA world began. The mobile, compartmentalized character of modern cells had yet to be invented. The active sites of modern enzymes still rely upon FeS- and NiS-type moieties; thus, it is likely that the early enzymes simply incorporated bits of their mineral heritage (see Section 7.1). Regarding encapsulation into membrane-bound compartments, experiments by D. Deamer in the 1970s showed that fatty acids have the capacity to self-assemble into membrane-like vesicles. More recently, Hanczyc et al. (2003) have demonstrated that conditions likely to prevail on an ancient seafloor (catalytic surfaces, hydrodynamic forces, alkaline conditions) have the potential to foster formation, growth, and division of fatty acid-based membranes that enclose biomolecules. Thus, the rudimentary mechanisms leading from surface-catalyzed proto-life to free-living cells seem to have been established (see Figure 2.3).

One potential weakness in the above-described "marine hydrothermal-vent mineral surfaces" hypothesis has been emphasized by Mukidjanian et al. (2012).These authors point out that cellular processes require inorganic mineral ions (especially K^+, Zn^{2+}, Mn^{2+}, and phosphate), yet the first cells possessed neither ion-tight membranes nor membrane pumps able to acquire and retain these essential mineral ions. For this reason, Mukidjanian et al. (2012) propose shallow terrestrial ponds of condensed geothermal vapor (underlain by porous minerals) as the most likely habitat for both pre-cellular metabolism and for the formation of phospholipid membranes, whose ion-tight nature would eventually allow encapsulated cells to spread and successfully colonize new geochemically dilute environments. Thus, the debate about the puzzle pieces of early life and how they fit together is ongoing (Arndt and Nisbet, 2012).

2.7 A PLAUSIBLE DEFINITION OF THE TREE OF LIFE'S "LAST UNIVERSAL COMMON ANCESTOR" (LUCA)

Molecular phylogenetic analyses of genes encoding small subunit ribosomal RNA (shared by all existing cellular life forms) have led to a tree of life with three primary domains, *Bacteria*, *Archaea*, and *Eukarya* (see Section 5.5). Insights from molecular phylogeny into evolutionary relationships and diversity are far-reaching. What is most germane to the present chapter is the root and main trunks of the tree of life. Perusal of the tree's base (Figure 2.6) shows a single point of bifurcation where the bacterial trunk diverges from the trunk destined to be the precursor of the other two domains. That point of divergence is conceptually profound – it represents the last universal common ancestor (LUCA). For the molecular phylogenist, the last universal common ancestor represents both an abstract idea and a tangible entity. This precursor to all life that we know today must have carried metabolic and evolutionary traits reflecting conditions and resources on ancient Earth. Indeed, by knowing physiological traits (thermophily, autotrophy, etc.) of today's organisms that reside near the base of the phylogenetic tree, inferences can be drawn about selective pressures of ancient life. The last universal common ancestor, spawned by the RNA world, is the endpoint of prebiotic evolution, which blossomed into organismic biology.

The last universal common ancestor was unlikely to have been a single well-defined entity. Rather, it would have been a community of precellular genetic complexes (Woese, 1998; Woese et al., 2000) that readily mixed their genetic and metabolic traits. In the model advanced by C. Woese, genuine organisms exhibiting distinctive lines of hereditary descent can only be found after the blurring effects of unrestricted gene transfer have largely ceased (Woese, 1998). Vertical gene transfer is heritage passed from parent to progeny. Lateral gene transfer is exchange of genetic material between forms of life that have become well-differentiated entities

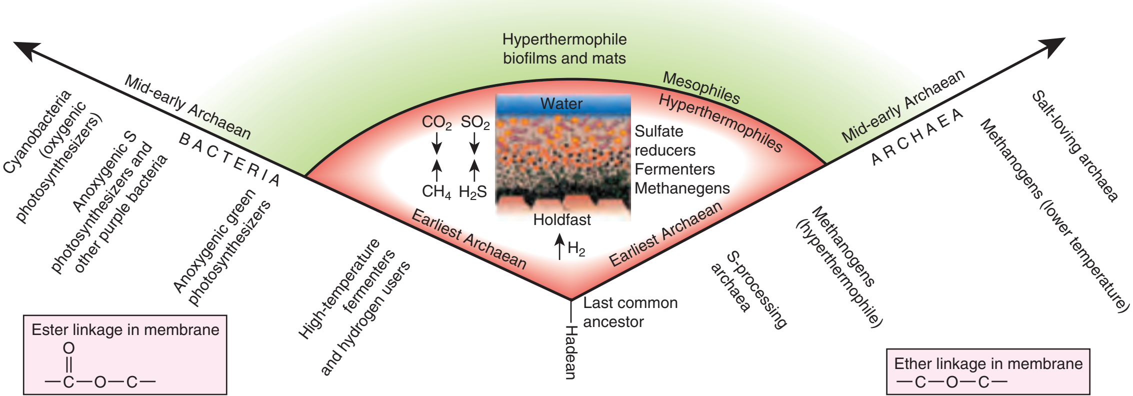


Figure 2.6 Divergence from the root of the tree of life: the last universal common ancestor. (Reprinted by permission from Macmillan Publishers Ltd: *Nature*, from Nisbet, E.G. and N.H. Sleep. 2001. The habitat and nature of early life. *Nature* **409**:1083–1091. Copyright 2001.)

(e.g., *Bacteria* and *Archaea*). The tools of molecular phylogeny examine patterns of heredity; therefore, such tools can only be applied after distinctive lines of descent have been established. Thus, the root, or origin, of the tree of life cannot be determined by ribosomal RNA sequences.

In the scenario developed by Martin and Russell (2003), the last universal common ancestor was the sophisticated precellular offspring of the RNA world that flourished in hydrothermal FeS cavities. Fundamental biochemical differences in the membranes of *Bacteria* and *Archaea* argue for *evolutionary divergence and encapsulation to be one and the same events* (Figure 2.6). The two earliest evolutionary lineages (*Bacteria* and the line that was to become *Archaea*) exhibit highly distinctive membrane lipid architecture: isoprenoid *ether-type* membranes in *Archaea* and fatty-acid *ester-type* membranes in *Bacteria* (Lombard et al., 2012). These are thought to have arisen in precellular life via repeated fissions and fusions of membranes of early cells, leading to proto-*Archaea* and proto-*Bacteria* with selective membrane biosynthetic capabilities. It is generally agreed that the *Archaea* and *Eukarya* arose from a second bifurcation in the trunk on the tree of life (see Sections 2.12 and 5.5). The presence of (seemingly a reversion to) ester-type membrane lipids in the *Eukarya* may have resulted from a merging of traits between an established bacterial line and a newly emerged eukaryal line – but the details of such developments are not yet clear (de Duve, 2007). Recently, Lombard et al. (2012) have assembled biochemical, phylogenetic, and genomic facts and principles aimed at explaining the bacterial-like nature of phospholipid membranes in *Eukarya*. Five separate evolutionary pathways were proposed (Lombard et al., 2012); judging one to be the most valid requires additional research.

Careful modeling of the molecular evolution of rRNA and associated proteins (Boussau et al., 2008) indicates that the last universal common

ancestor was a mesophile (favoring ~50 °C temperatures) and that both bacterial and archael lines of descent subsequently adapted to higher (thermophilic) temperatures.

2.8 THE RISE OF OXYGEN

Regarding biological complexity, defining "here" (modern life), "there" (a sterile prebiotic Earth), and chronicling likely mechanistic evolutionary connections between the two is an ongoing challenge (see Sections 2.4 to 2.7). An analogous challenge confronts atmospheric chemists whose goal is to understand the transition between a highly reducing, abiotic Earth 4.6×10^9 years ago and the highly oxidizing, biotic Earth of today. The rise of biota and the rise of oxygen in Earth's atmosphere (from 0% to 21%) went hand in hand. Analogous to scenarios woven to explain the evolution of modern life, there are several key facts (milestones, benchmarks) that are critical for understanding the oxygenation of planet Earth. These benchmarks include: ancient evidence of oxygen as oxide minerals in the sedimentary record, the photosynthetic mechanism of oxygen production, and the consequences of oxygen production – including an atmospheric ozone shield, aerobic metabolism, and the appearance of higher eukaryotes late in evolution (see Table 2.1 and Figure 2.2).

2.9 EVIDENCE FOR OXYGEN AND CELLULAR LIFE IN THE SEDIMENTARY RECORD

In his eloquent portrayal of early Earth, A. Knoll (2003) describes P. Cloud's efforts to identify a demarcation line in the sedimentary record (between 2.4 and 2.2×10^9 years ago) for the appearance of oxygen on our planet. The iron sulfide mineral, pyrite (FeS_2), is thermodynamically unstable in the presence of molecular oxygen, particularly during cycles of erosion and deposition. Sedimentary rocks have been surveyed globally and those younger than 2.4×10^9 years generally do not contain pyrite while many older ones do. This argues for the absence of widespread atmospheric oxygen before 2.4×10^9 years ago. Evidence for the presence of atmospheric oxygen after 2.2×10^9 years ago resides in the formation of iron oxide minerals. These are manifest as "red beds" such as the vividly colored red rock formations in sedimentary successions visible today in Utah and Arizona, western United States (see Figure 2.1).

Another important line of evidence in deciphering the history of oxygen has been provided by J. Farquhar. Prior to being transformed and deposited in sediments, sulfur dioxide, an atmospheric gas, interacts with sunlight in the atmosphere, leaving a characteristic signature in the sulfur isotopic ratios. If molecular oxygen is present, the characteristic isotopic ratios are erased. According to Kerr (2005), Farquhar and colleagues have

found the sulfur signature in rocks older than 2.4×10^9 years, but not in younger rocks.

While the vast red bed formations and related facts argue convincingly for a widespread oxygenated atmosphere 2.4–2.2×10^9 years ago, this does not preclude the possibility of small-scale hot spots of oxygen production earlier. H. Ohmoto has reported the occurrence of oxidized minerals as old as 3×10^9 years. It is possible that these represented localized islands of oxygen-producing biota surrounded by confining sinks for oxygen. Molecular oxygen spontaneously reacts with (oxidizes) many reduced chemical compounds (e.g., H_2S, Fe^{2+}) that prevailed in the Proterozoic era. This means that appreciable atmospheric oxygen concentrations could only have developed after such oxygen sinks were depleted. Indeed, it has been argued that early oxygen in the atmosphere hovered between 1% and 10% for as long as 1.8×10^9 years after oxygenic photosynthesis evolved. At these low concentrations, oxygen would not be expected to penetrate the deep oceans. D. Canfield and A. Knoll have interpreted the sulfur isotopic record as indicating that all but the upper oceanic layers were anoxic long after oxygen began creating the red bed deposits. Isotopic ratios of molybdenum, also an indicator of oxygen concentration, have confirmed widespread hypoxia in the early oceans.

The era of the sulfur-dominated Canfield Ocean ended by 0.6×10^9 years ago when atmospheric oxygen reached its current atmospheric concentration (21%). Several theories have been offered to explain why oxygen accumulated so significantly. Oxygenic photosynthesis is directly linked to conversion of CO_2 to organic carbon in biota. In a steady-state condition, oxygenic photosynthesis is balanced by the reverse reaction: aerobic respiration by heterotrophic life of fixed organic carbon. Oxygen's rise in the atmosphere hinged upon a major shift in the balance between the two processes. Vast masses of plant biomass (organic carbon) were generated and subsequently buried in ocean sediments. As this organic carbon entered long-term storage in sedimentary basins (sequestered from the biosphere), oxygen concentrations climbed. One potential geochemical explanation for carbon sequestration is its reaction with seafloor clays that may have been capable of protecting organic matter from respiration by heterotrophic microorganisms. Another geochemical explanation favors plate tectonic formation of a supercontinent that relieved nutrient limitations in the ocean, thereby stimulating carbon fixation and burial. Potential biological explanations for increased photosynthetic activity and carbon burial include: (i) the evolution of land-based lichens that may have boosted fluxes of otherwise limiting nutrients to the oceans and (ii) the evolution of zooplankton, whose carbon-rich fecal pellets sank rapidly to the ocean floor.

Table 2.2 lists "paleontology" and the "fossil record" as valuable sources of information about early Earth. We know that skeletons of many animals are well preserved in geologic strata. It seems prudent to seek corresponding preserved structures for prokaryotic life. Among the most robust evidence

(a)

(b)

Figure 2.7 Examples of both modern (a) and ancient (b) microbial mats. (a) Contemporary stromatolites in Shark Bay, Western Australia (Wikipedia). (b) Stromatolite fossil aged 2.5×10^9 years from the Barberton Mountains, South Africa. Note the scale: the visible rock face in this image is 20 cm wide by 17 cm tall. (With permission from David J. Des Marais, NASA Ames Research Center.)

for fossil prokaryotic structures are stromatolites. Stromatolites are macroscopic layered microbial communities that grow today in shallow coastal waters such as those in northwestern Mexico and Western Australia (Figure 2.7a). Filamentous cyanobacteria contribute significantly to these microbial mat (stromatolite) ecosystems. Fossilized stromatolites have been found on many continents and are well represented in samples from South Africa's Barberton Mountains, dated 2.5×10^9 years old (see Figures 2.1 and 2.7b). The Australian Pilbara chert deposits (3.4×10^9 years old) have revealed unusual forms of stromatolite-like structures (Allwood et al., 2006, 2009). Smaller-scale bacteria-like structures have been reported in some ancient rocks, such as the 3.5×10^9-year-old pillow lavas in the Barberton Greenstone Belt in South Africa and in the Warrawoona Group, Western Australia (Shopf et al., 1987; Shopf, 1988). Recent microscopic and mass spectrometric evidence link pyrite formation to microbial S transformations (oxidation of elemental sulfur and sulfate reduction) 3.4×10^9 years ago (Wacey et al., 2011).

2.10 THE EVOLUTION OF OXYGENIC PHOTOSYNTHESIS

D. Des Marais (2000) points out that one key index of life and its success is the accumulating mass of fixed carbon, otherwise known as biomass (see Section 2.9). Biogeochemically, it is reducing power that provides a means of converting the pool of inorganic oxidized carbon (CO_2) to the organic form that constitutes living organisms. When CO_2 is converted by autotrophic life forms to organic carbon (CH_2O), the carbon is reduced (accepts electrons) from an oxidation state of +4 to 0 (see Section 3.6 and Box 3.4; see also Sections 7.3 and 7.4). Geochemical sources of reducing power in early Earth were limited largely to hydrothermal sources of H_2S, Fe^{2+}, Mn^{2+}, H_2, and CH_4. These only occurred at relatively low concentrations across the globe. However, another vast pool of potential reducing power was there *if it could be tapped*: water. The supply of water is virtually unlimited – it occurs at a concentration of 55 M. If a sun-driven biochemical mechanism evolved to use the atoms of oxygen in water as a source of electrons ($H_2O \rightarrow {}^1/_2O_2 + 2H^+ + 2e^-$), biomass formation and life's colonization of Earth habitats would accelerate.

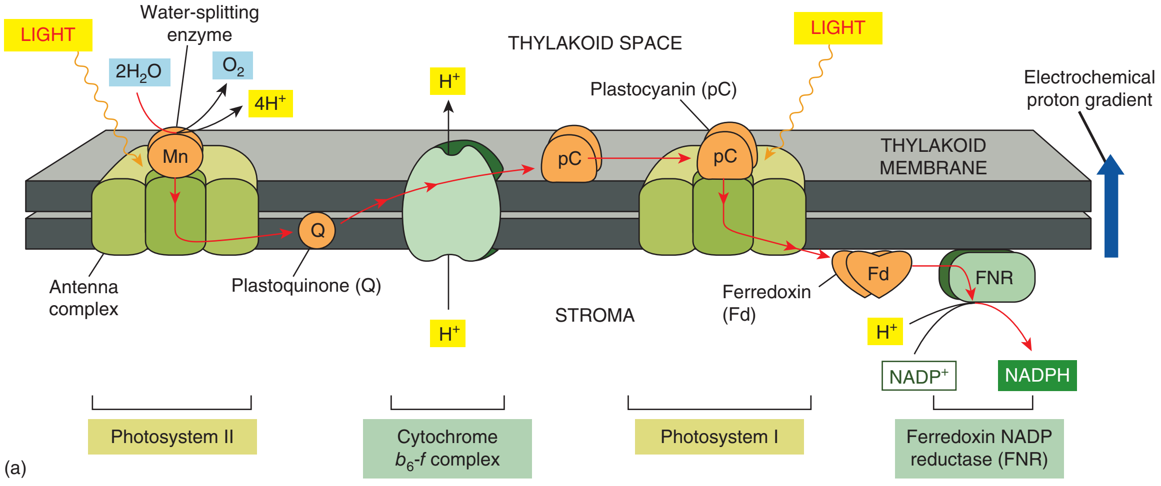
Where did oxygenic photosynthesis come from? It features a complex, membrane-bound electron-transport assembly that includes chlorophyll, two light reaction centers, a manganese-containing water-splitting enzyme, a cytochrome proton pump, a ferredoxin NADP (nicotinamide adenine dinucleotide phosphate) reductase, ATP synthase, and light-harvesting pigments. Overall, light is harvested and used to steal electrons from water molecules and then electrons are funneled through transport chains to generate ATP and to reduce CO_2. Perhaps predictably, the elegant contemporary oxygenic photosynthesis apparatus (Figure 2.8) is resolvable into a series of components that each has its own origin and evolution

(Knoll, 2003; Olson and Blankenship, 2004; Hohmann-Marriott and Blankenship, 2011). Remarkably, a primitive version of this apparatus was present in representatives of cyanobacteria 2.7×10^9 years ago (see Table 2.1, Figure 2.2, and Box 2.2).

The fundamental functional unit of photosynthesis is the "reaction center" (RC) (Olson and Blankenship, 2004; Hohmann-Marriott and Blankenship, 2011). Biochemically, RCs are membrane-bound associations of porphyrin-containing chlorophyll molecules, proteins, and light-harvesting pigments. The deep roots of RCs are unclear – they may have had an early precellular (last universal common ancestor) phase, based on the aqueous chemistry of porphyrins. Alternatively, the earliest RCs may have developed from early membrane-bound respiratory cytochrome proteins.

The oxygenic photosynthetic apparatus of modern cyanobacteria and higher plants is depicted in Figure 2.8. In the classic "Z scheme" of electron flow (a "Z" on its side), light energy activates a manganese-containing RC (photosystem II), which liberates electrons from water, generating molecular oxygen and protons. Electrons are then carried from a reduced (activated) chlorophyll molecule through a series of quinones, and cytochromes to a protein that donates the electrons to another RC (photosystem I), whose chlorophyll catalytic site contains magnesium. The electrons are accepted by the RC chlorophyll of photosystem I, which initiates light-activated transfer of electrons through several carriers, leading to the production of reducing power ($NADP^+$). Thus, modern oxygenic photosynthesis is accomplished by two different photosystems linked together. During flow of an electron from the RC in photosystem II to the RC in photosystem I, the energy state of the electron is boosted twice by light quanta, and after each boost electron transport flows in a thermodynamically favorable (negative to positive) direction. This generates both reducing power and a proton motive force (see Section 3.6) that allows ATP synthase to form ATP. The reducing power and ATP can then fuel the reduction of CO_2 and formation of biomass, often through the Calvin cycle via the enzyme ribulose bisphosphate carboxylase (see Section 7.4). Because CO_2 fixation is independent of light, this process is often referred to as "dark reactions".

The modular nature of oxygenic photosynthesis is obvious: two separate distinctive photosystems (with their respective RCs) complement one another. In Olson and Blankenship's (2004) essay summarizing current thought on the evolution of photosynthesis, the authors admit that certainty about ancient events is unlikely to ever be achieved, but they have prepared a feasible scenario drawing evidence from geology, biogeochemistry, comparative biochemistry, and molecular evolution. This scenario presents a model showing how the genotype (and phenotype) of a primordial RC may have developed into the three RC types represented in contemporary photosynthetic prokaryotes: (i) the (anoxygenic) purple nonsulfur and green nonsulfur bacteria; (ii) the (anoxygenic) green sulfur bacteria, *Heliobacteria*, and *Acidobacteria*; and (iii) the oxygenic cyanobacteria



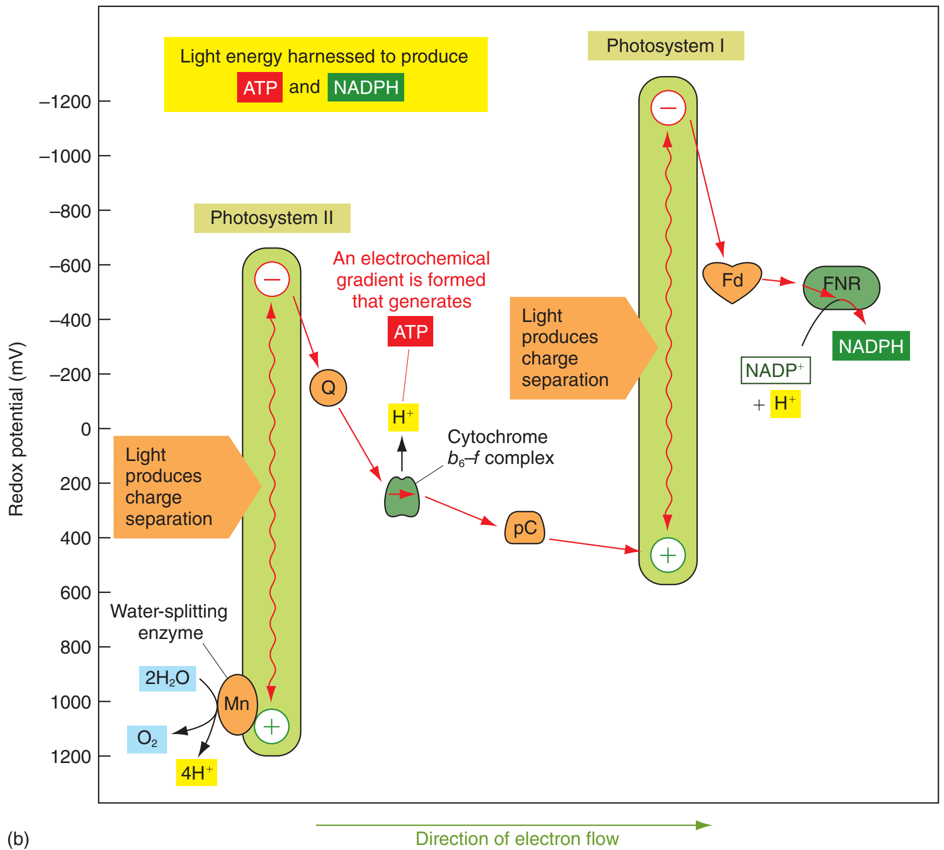


Figure 2.8 Functional scheme for electron flow in oxygenic photosynthesis in cyanobacteria and in higher plants. (a) Three-dimensional arrangement of photosystem components in their supporting membrane structure. (b) Diagram of energy and electron flow through photosystem II (left) to photosystem I (right). For a further explanation, see the text. (From Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. *Molecular Biology of the Cell*, 4th edn. Garland Science Publications, Taylor and Francis Group, New York.)

and chloroplasts (Figure 2.9). In the proposed scenario, the ancestral RC diverged twice, creating two pairs of RCs – each featuring different electron-accepting proteins: pheophytin-quinone or iron-sulfur. A sequence of gene duplication and protein fusion events is thought to have combined the pheophytin-quinone RC with manganese-dependent catalase to create photosystem II, which used light energy to oxidize water. An early cyanobacterial cell was host to both types of RC. In this context, oxygenic, "Z scheme" photosynthesis may have been invented. One clear inference from Figure 2.9 is that photosystem II developed late – it was preceded by many prior RC combinations in many hosts. The recently published update on photosynthesis' evolution (Hohmann-Marriott and Blankenship, 2011) reiterates the developmental events shown in Figure 2.9; however, additional details about molecular structures of RCs are provided.

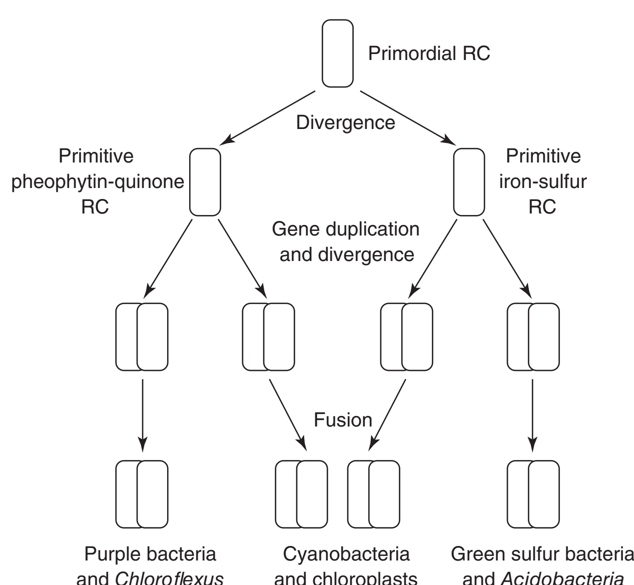


Figure 2.9 Evolution of photosynthetic reaction centers (RCs) that are the basis for photosystems I and II. Contemporary agents of oxygenic photosynthesis (cyanobacteria and chloroplasts) host photosystems I and II. (From Olson, J.M. and R.E. Blankenship. 2004. Thinking about the evolution of photosynthesis. *Photosynthesis Res*. **80**:373–386, fig. 4, p. 379. With kind permission of Springer Science and Business Media.)

As mentioned above, typical anoxygenic photosynthetic bacteria of today include purple bacteria, green sulfur bacteria, *Acidobacteria*, and *Heliobacteria*, whose electron donors range from H_2S to $S_2O_3^{2-}$, S^0, or Fe^{2+}. Owing to the relatively large pool of Fe^{2+} in ancient oceans, it is widely accepted that the ancient anoxygenic photosynthesis was responsible for oxidizing Fe^{2+} to Fe^{3+}. Insoluble Fe^{3+} precipitated and was deposited on seafloor beds, perhaps creating the banded iron formations (BIFs) prevalent in the global sedimentary record – as early as 3.7×10^9 years ago (Olson and Blankenship, 2004). The alternative hypothesis for BIFs is direct chemical oxidation of Fe^{3+} and Fe^{2+} by molecular oxygen (Hohmann-Marriott and Blankenship, 2011).

2.11 CONSEQUENCES OF OXYGENIC PHOTOSYNTHESIS: MOLECULAR OXYGEN IN THE ATMOSPHERE AND LARGE POOLS OF ORGANIC CARBON

Figure 2.10 uses a clock-like metaphor to depict the chronology of oxygen-related and other events on life's development on Earth. The great inventors, oxygen-producing cyanobacteria, began their transformation of the biosphere about 2.7×10^9 years ago. The light-driven photosynthetic

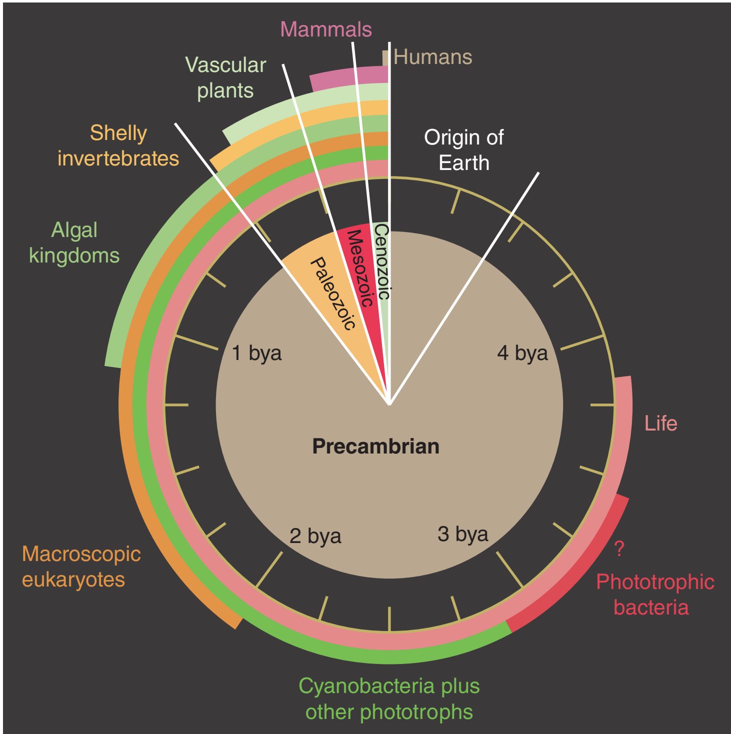


Figure 2.10 Earth's biological clock; bya, billions of years ago. (From Des Marais, D.J. 2000. When did photosynthesis emerge on Earth? *Science* **289**:1703–1705, with permission.)

apparatus created atmospheric oxygen – simultaneously providing ATP and reducing power to fix CO_2 into organic carbon. The manifestation of oxygenic photosysnthesis, light reactions making oxygen plus ATP, and dark reactions making biomass, impacted the Earth and its inhabitants via at least five different pathways (Figure 2.11). In the physiological roles of an electron donor and carbon source (see Section 3.3), the reduced organic carbon fueled biochemical innovation for both prokaryotic and eukaryotic heterotrophic life. Moreover, the molecular oxygen had drastic geochemical effects displayed in the sedimentary record and also fostered biochemical innovation in prokaryotic and eukaryotic physiology. Large ATP yields became available to organisms utilizing oxygen-dependent respiratory chains (see Sections 3.8 and 3.9). This allowed a "Cambrian explosion" in biological diversity – especially among eukaryotes (see Figure 2.2). The molecular oxygen also formed an ozone shield that curtailed damage to biota by UV radiation and allowed life's transition to new ecological niches on land.

There are physiological drawbacks to oxygen, however. Its metabolic byproducts can be toxic. Figure 2.12 provides a summary of the many chemical transformations that molecular oxygen (known as "triplet" or "ground state" oxygen) can routinely undergo during the reduction of O_2 to H_2O in aerobic respiration. Toxic, transient forms of oxygen include singlet oxygen, superoxide radical, and hydrogen peroxide. Without protection from these reactive oxygen species (ROS), the structure of essential cellular components (e.g., DNA, lipids, proteins, and carbohydrates) can be severely altered. The result can be failure in membrane and reproductive function. The many innovations of aerobic life could never have developed without the evolution, in parallel, of both enzymatic and nonenzymatic metabolic systems that eliminated ROS. Well-known enzymatic ROS-elimination systems include catalase (converting H_2O_2 to H_2O and O_2), peroxidase (converting H_2O_2 to H_2O), superoxide dismutase (converting O_2^- to H_2O_2 and O_2), and superoxide reductase (converting O_2^- to H_2O_2). Nonenzymatic ROS scavengers

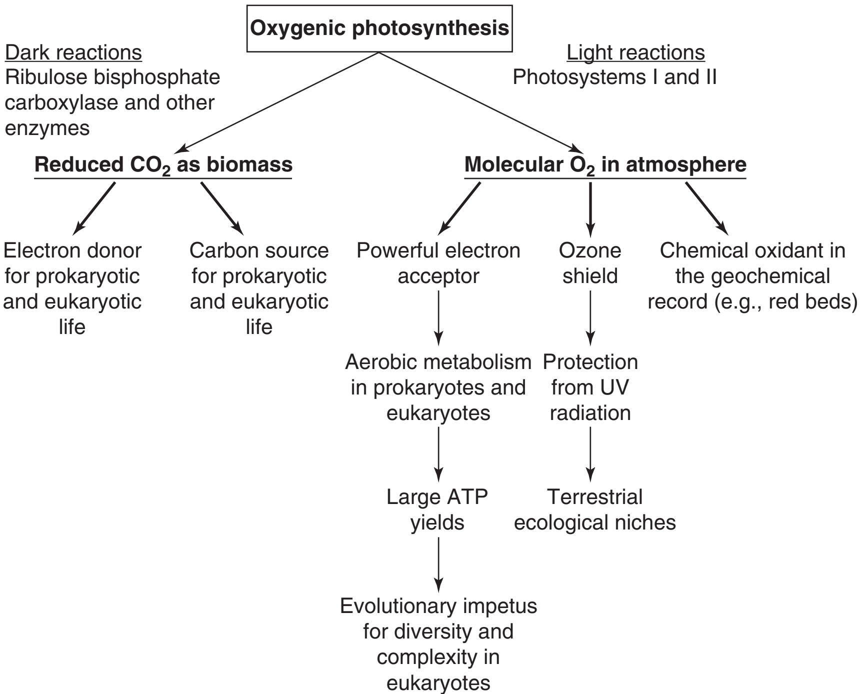


Figure 2.11 Five major chemical, physiological, and evolutionary impacts of oxygenic photosynthesis.

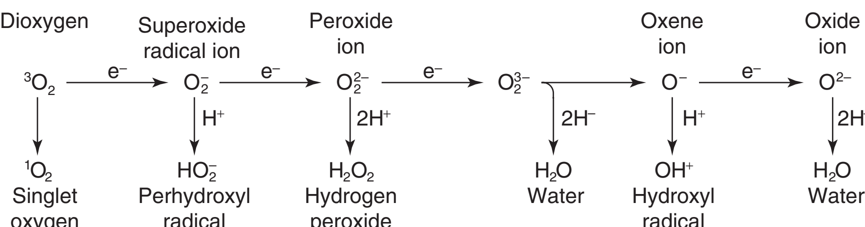


Figure 2.12 Oxygen's toxic downside: generation of reactive oxygen species (ROS) by common energy transfer reactions in cells. (From Rodriguez, R. and R. Redman 2005. Balancing the generation and elimination of reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **102**:3175–3176. Copyright 2005, National Academy of Sciences, USA. Original source: Apel, K. and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55: 373–399, reprinted with permission from Annual Reviews.)

include glutathione and proline. ROS are unavoidable byproducts of biochemical pathways (such as respiration, glycolysis, and photosynthesis) that are central to energy production and storage strategies of aerobic microorganisms, animals, and plants. Thus, the growth and reproduction of all aerobic life is a balancing act between the generation of ROS and the capacity of antioxidation systems to eliminate ROS.

As the Earth's pool of molecular oxygen gradually grew, the biosphere's original prokaryotic life forms that flourished in the absence of oxygen were forced to adapt. They did so either by acquiring oxygen-defense mechanisms (via direct evolution or lateral gene transfer from other organisms) or by receding into refuges protected from toxic oxygen. Earth features many habitats (e.g., waters, soils, sediments, gastrointestinal tracts of animals) where oxygen fails to penetrate. Oxygen-free locales reflect the dynamic balance between the rate of oxygen influx by diffusion/convection and the rate of consumption by oxygen-respiring microbial communities. Respiratory demand by the microorganisms dwelling in a thin layer of mud at the sediment–water interface in lakes, streams, and the ocean can easily outpace the rate of oxygen influx. This is because oxygen has limited solubility in water (~9 mg/l at 20 °C when oxygen is 21% of the atmosphere) and oxygen diffuses into water from the atmosphere quite slowly.

2.12 EUKARYOTIC EVOLUTION: ENDOSYMBIOTIC THEORY AND THE BLENDING OF TRAITS FROM *ARCHAEA* AND *BACTERIA*

The tree of life is a portrait of evolution based on molecular phylogeny of genes encoding small subunit ribosomal RNA (see Sections 2.7 and 5.5). The three-domain concept provides a framework for refining hypotheses about how life developed. If developments were based solely upon linear evolutionary trajectories, once a new positive trait arose, it might be expected to remain exclusively within its original line of descent (see Section 5.9). In practice, however, phylogenetic trajectories often depart from linearity. This departure probably is caused by two major factors: (i) genes being transferred laterally between lines of descent and (ii) fluctuating environmental conditions (hence selective pressures), which over the eons foster hereditary discontinuities, via loss of genes and/or their host via extinction.

Insights into the robustness of the tree of life can be obtained by examining other traits (morphological, genetic, biochemical) carried by current members of the *Bacteria*, *Archaea*, and *Eukarya*. Table 2.3 provides a comparison of such traits across the three domains of life. The traits listed in Table 2.3 include overall cell structure, chromosome structure, the presence of a nucleus, the presence of organelles, membrane structure, the structure of ribosomes, gene organization, mechanisms of protein synthesis, RNA biochemistry, sensitivity to high temperature, and metabolic diversity. The pattern of traits in the table clearly supports the broad message from the tree of life: *Bacteria*, *Eukarya*, and *Archaea* are distinctive, though *Archaea* have commonalities with the other two domains. Refined phylogenetic results and biochemical correlations have shown that the

Table 2.3

A comparison of morphological and biochemical traits of *Bacteria*, *Archaea*, and *Eukarya*. (Modified from both Madigan et al., 2012, and White et al., 2012)

Trait	*Bacteria*	*Archaea*	*Eukarya*
Prokaryotic cell structure	+	+	–
Chromosomal DNA in closed circle	+	+	–
Histone proteins with DNA	–	+	+
Nucleus	–	–	+
Mitochondria and/or chloroplast organelles	–	–	+
Cell wall with muramic acid	+	–	–
Membrane lipids	Ester-linked	Ether-linked	Ester-linked
Ribosome mass	70S	70S	80S
Introns in most genes	–	–	+
Initiator tRNA	Formyl methionine	Methionine	Methionine
RNA polymerase	One (5 subunits)	Several (8–12 subunits each)	Three (12–14 subunits each)
Genes as operons	+	+	–
mRNA tailed with polyA	–	–	+
Sensitivity to antibiotics (chlorophenicol, streptomycin, kanamycin) that increase errors during protein synthesis	+	–	–
Growth above 70 °C	+	+	–
Growth above 100 °C	–	+	-
Chemolithotrophy	+	+	–
Nitrogen fixation	+	+	–
Denitrification	+	+	–
Dissimilatory reduction of SO_4^{2-}, Fe^{3+}, Mn^{4+}	+	+	–
Methanogenesis	–	+	–

genetic lines of *Eukarya* and *Archaea* have a common ancestral branch that is independent of the one that gave rise to *Bacteria*. Thus *Eukarya* and *Archaea* are more closely related to one another than either is to *Bacteria*. The biochemical, physiological, and genomic characteristics of the three domains of life are extensively discussed by Madigan et al. (2014) and White et al. (2012).

The hypothetical route from simple precellular life to the advanced multicellular eukaryotes of today is presented in Figure 2.13. After *Bacteria*

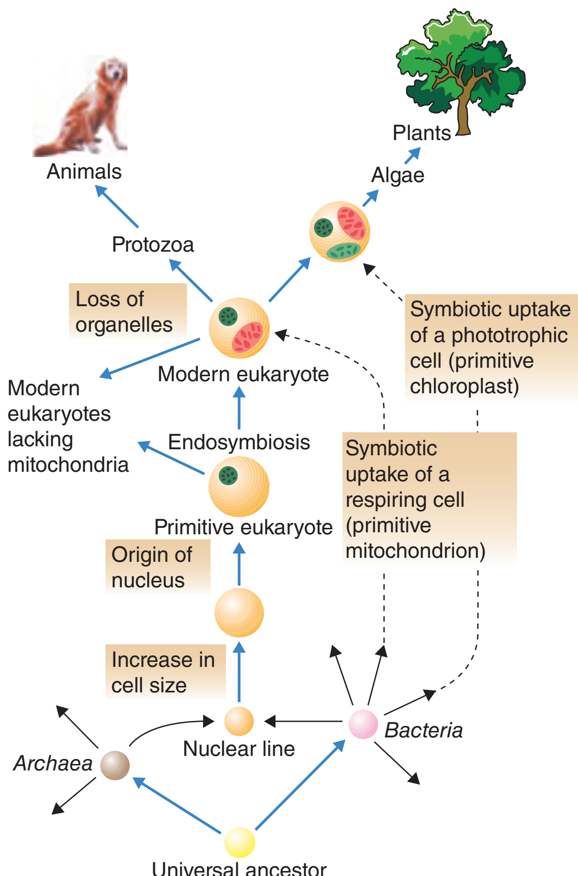


Figure 2.13 Hypothetical evolutionary events leading from the last universal common ancestor through the three domains to the endosymbiotic production of modern eukaryotes and their developmental achievements. (Modified from Madigan, M. and J. Martinko, 2006. *Brock Biology of Microorganisms*, 11th edn, p. 307. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ.) Primitive eukaryote (nucleated cells) likely date back to 3.5×10^9 years ago (Table 2.1).

and *Archaea* diverged from the last universal common ancestor, the third domain (Eukarya) is thought to have arisen via a subsequent fusion of bacterial and archaeal lines. The *Eukarya* developed a membrane-bound nucleus to accommodate an expanded genome, which contributed to increased cell size. The eukaryotic nuclear membrane and other membrane systems may be the result of early, poorly regulated expression of bacterial membrane replication: vesicles may have formed and fortuitously accumulated in the cytosol near their site of synthesis.

Endosymbiosis, as advanced by L. Margulis in the 1960s, is likely to have played a crucial role in eukaryotic development (see Section 8.1). Mitochondria, the ATP-generating organelles carried almost universally by *Eukarya*, feature cell structures (membranes, 16S RNA not 18S rRNA) that unquestionably are of bacterial origin. In fact, the 16S rRNA gene sequences of mitochondria are closely related to α-Proteobacteria. This suggests that symbiotic uptake by a unicellular eukaryote allowed an originally free-living aerobic bacterium to evolve into mitochondria by specializing in ATP generation (Figure 2.13). Similarly, the photosynthetic organelles of green plants (chloroplasts) feature undeniable bacterial characteristics and a 16S rRNA gene sequence closely related to that of cyanobacteria. Endosymbiotic cyanobacteria became chloroplasts. Accordingly, algae and higher plants of today appear to be the product of two sequential endosymbiotic events (Figure 2.13).

To close this section and chapter, it seems prudent to develop a rudimentary sketch of the key characteristics of microorganisms. The six contemporary types of microorganisms are *Bacteria*, *Archaea*, fungi, protozoa, algae, and viruses. Information in Box 2.3 solidifies major themes about the structural distinctions between prokaryotic and eukaryotic forms of life and how microbiologists do their work. Additional details on the diverse cellular and physiological traits of microorganisms appear in Chapters 3, 7, and 8, and particularly in Chapter 5.

Box 2.3

A primer on what microorganisms are and how they are studied: small size scale makes a big difference for microbiologists

When cataloging the characteristics of creatures, all biologists seek and find unifying themes and fascinating contrasts in the structure, behavior, and ecology of their objects of study. Thus, there are many commonalities between how microbiologists study microorganisms and how plant and animal biologists study plants and animals. However, unlike plants and animals, individual microorganisms (*Bacteria*, *Archaea*, protozoa, fungi, algae, and viruses) can be seen only with a microscope. The size of prokaryotic cells (*Bacteria* and *Archaea*) is about 1 μm. Shapes of individuals include spheres (cocci), rods, or spiral forms; these may be in clusters, chains, or long filaments.

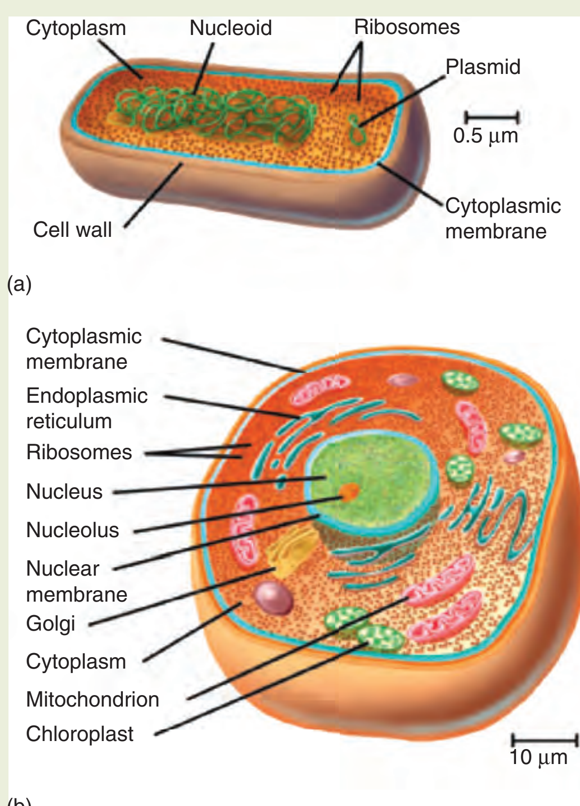


Figure 1 Comparison of key structural features of (a) prokaryotic and (b) eukaryotic cells. (From Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms,* 11th edn, p. 22. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ.)

Except for viruses (which are intracellular parasites; see Section 5.8), within each unicellular microorganism are all of the genetic and biochemical structures that allow metabolism and self-replication to occur. Growth of microbial cells relies upon the coordination of approximately 2000 chemical reactions that both generate energy (especially as ATP) and utilize that energy in biosynthetic production of all the materials (membranes, cell walls, proteins, DNA, RNA, ribosomes, etc.; see also Sections 3.3 and 3.9) needed to build new organisms. Figure 1 provides a generic overview comparing prokaryotic and eukaryotic cells.

As is clear from Figure 1 in this box and information in Table 2.3, major contrasts between prokaryotic microorganisms (*Bacteria* and *Archaea*) and eukaryotic microorganisms (fungi, algae, protozoa) include size (~1 μm versus >10 μm, respectively), the organization of genetic material (as nucleoid and plasmid DNA versus a membrane-enclosed nucleus, respectively), and the presence of other specialized membrane-enclosed organelles (e.g., mitochondria and chloroplasts) only in eukaryotes.

While "macrobiologists" can inspect an individual eagle or flower or aardvark to

Box 2.3 ***Continued***

gain deep insight into the structure and function of each creature, microbiologists often take a "collective" approach to studying microbial biology and taxonomy. Microbiologists routinely rely upon growing and isolating colonies of a single (purified) microorganism on solid media (e.g., agar) in the laboratory. Colonies of microorganisms are composed of thousands to millions of cells derived from a single cell that grew exponentially, via binary fission, on nutrients provided by the solid medium. During binary fission, all cell components (DNA, ribosomes, protein complexes, inorganic ions, etc.) essentially double within an elongating mother cell. After partitioning of the intracellular materials, a septum forms in the middle of the elongated cell. Next, completion of new cell wall synthesis at the septum allows the two daughter cells to separate and be released.

Operationally, a pure culture of a given microorganism is one that exhibits consistent phenotype and genotype after sequential subculturing (it must "breed true"). Subcultures of purified microorganisms can be inoculated into many types of liquid or solid media and the abilities of ~10^9 individual cells to grow and/or produce particular metabolic byproducts (acids, gases, metabolites) can be assessed. For microbiologists, these abilities [to grow (or not grow) and produce (or not produce) metabolites] constitute key phenotypic and genotypic information. Such information is analogous to leaf shape and flower morphology – used by botanists to classify plants. One widely used phenotypic trait for classifying *Bacteria* is the Gram stain. In 1884, Christian Gram discovered a fundamental structural distinction between two main types of bacterial cells. When stained with crystal violet and iodine, Gram-positive cells (with a thick multilayered peptidoglycan cell wall) retain crystal violet and its purple color after being rinsed with alcohol. However, Gram-negative cells (with thin peptidoglycan walls and lipid-rich outer membranes) are decolorized by alcohol.

Chapter 5 conveys a substantial amount of additional information about microorganisms and microbial diversity. Within Chapter 5, Section 5.6 provides a sampling of the traits of many prokaryotic and eukaryotic microorganisms and their phylogenetic relationships.

STUDY QUESTIONS

1 Devise a way to test the "panspermia hypothesis". To do this, presume that you have access to samples from many planets near Earth and access to all tools listed in Table 2.2. Begin by defining the logic that you would use. Then state what measurements would be performed on which samples and how you would interpret the data.

2 Table 2.1 indicates that the early surface of Earth was subjected to extreme conditions. What habitat might have been spared these extremes? Might these habitats have been preferred sites for life's early stages?

3 *^{14}C dating of a peat core*. You are studying the microbiology of peat bogs. A core of peat is retrieved from a depth of 6 m and you want to know the age of the preserved plant material. Presume that, since being originally deposited, there has been no replenishment of ^{14}C from the atmosphere. Thus, the amount of ^{14}C there is the residual since radioactive decay began. The measurements you complete reveal that the ^{14}C content is 0.03125 times that of the surface material.

How many half-lives have passed? How old is the material? Why is the limit for dating based on radioactive decay limited to about 10 half-lives? (Hint: use information in Box 2.1 to answer this question.)

4 Wachtershauser's theory of early metabolism relies on the notion that mineral surfaces at the seafloor–water interface catalyzed chemical reactions. Are there mineral surfaces today that catalyze chemical reactions? If so, please provide some examples. (To answer this, search library sources and/or the world wide web.)

5 Section 2.6 describes a hypothesis that competes with oceanic hydrothermal vents as the habitat where metabolism and cellular life began. Do you favor the "shallow terrestrial geothermal pond" hypothesis? If so, why? If not, why not?

6 The last universal common ancestor is an intriguing concept. The genetic heritage that you share with your siblings (plus parents, grandparents, and great grandparents) establishes a "line of descent". Name one major factor that is absolutely necessary for lines of descent to be traced. Name another factor that would readily "blur" the lines. Briefly explain both factors.

7 Why are cyanobacteria (and their ancestors) the "great innovators" of evolution? List and explain six major impacts (direct or indirect) that cyanobacteria had on evolution.

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3

Physiological Ecology: Resource Exploitation by Microorganisms

This chapter begins by presenting an important concept: Habitats provide selective pressures over evolutionary time. *Thus, facts and principles in Chapters 3 and 4 (a survey of Earth's habitats) are intimately linked. Next, we develop a genome-based definition of microorganisms and review universal functional categories of cellular processes. This is followed by discussion of habitat-specific factors that are likely to have shaped microbial evolution: nutrient availability and a need for dormancy. Conditions for life on Earth are then presented as "mixtures of materials in chemical disequilibrium". Thermodynamics provides a way to systematically organize and quantify the many biogeochemical reactions that microorganisms catalyze. The chapter ends by discussing metabolism, the logic of electron transport reactions for adenosine triphosphate (ATP) production, and the diversity of lithotrophic metabolic reactions.*

Chapter 3 Outline

3.1 The cause of physiological diversity: diverse habitats provide selective pressures over evolutionary time
3.2 Biological and evolutionary insights from genomics
3.3 Fundamentals of nutrition: carbon- and energy-source utilization provide a foundation for physiological ecology
3.4 Selective pressures: ecosystem nutrient fluxes regulate the physiological status and composition of microbial communities
3.5 Cellular responses to starvation: resting stages, environmental sensing circuits, gene regulation, dormancy, and slow growth
3.6 A planet of complex mixtures in chemical disequilibrium
3.7 A thermodynamic hierarchy describing biosphere selective pressures, energy sources, and biogeochemical reactions
3.8 Using the thermodynamic hierarchy of half reactions to predict biogeochemical reactions in time and space
3.9 Overview of metabolism and the "logic of electron transport"
3.10 The flow of carbon and electrons in anaerobic food chains: syntrophy is the rule
3.11 The diversity of lithotrophic reactions

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3.1 THE CAUSE OF PHYSIOLOGICAL DIVERSITY: DIVERSE HABITATS PROVIDE SELECTIVE PRESSURES OVER EVOLUTIONARY TIME

Chapter 2 presented the conditions of early Earth and the transition from its abiotic past to its biotic present. Metabolism, replication, and heredity are critical traits of life. These three processes are inseparable from the environmental context that provides resources for metabolism and selective pressures for both replication and heredity. Figure 3.1 conceptually depicts the long (3.8×10^9 years) and dynamic dialog between Earth's habitats and microorganisms. The dialog is framed by thermodynamics because, by systematically examining thermodynamically favored geochemical reactions, we can understand and predict selective pressures that act on microorganisms. There is a mechanistic series of linkages between our planet's habitat diversity and what is recorded in the genomes of microorganisms found in the world today. Diversity in habitats is synonymous with diversity in selective pressures and resources. When operated upon by forces of evolution, the result is molecular, metabolic, and physiological diversity found in extant microorganisms. This chapter will focus on the microbiological component (nutritional, genomic, biochemical, and physiological; upper right-hand sphere of Figure 3.1) and Chapter 4 will focus on the broad catalog of Earth habitats (upper left-hand sphere of Figure 3.1).

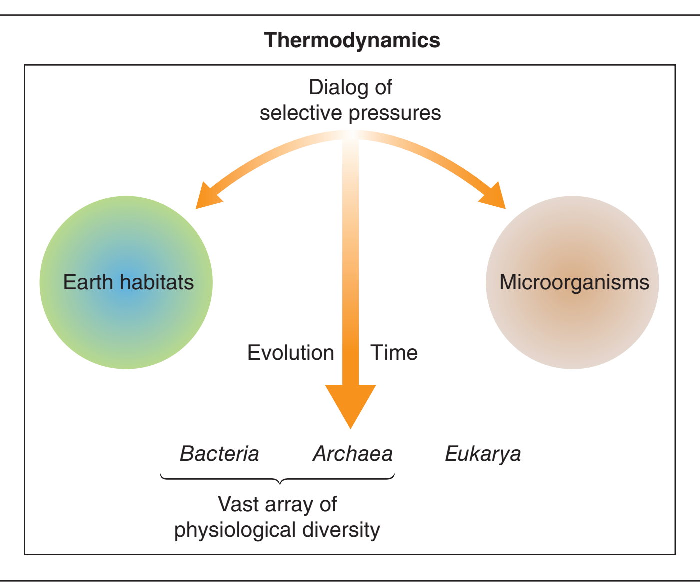


Figure 3.1 Conceptualized mechanism for the development of past and current microbial metabolic diversity: diverse habitats provide selective pressures over evolutionary time.

3.2 BIOLOGICAL AND EVOLUTIONARY INSIGHTS FROM GENOMICS

Genome size

To begin to discern what prokaryotes are and the evolutionary pressures that have shaped them, we need only turn to the ongoing revolution in biology: genomics (e.g., Fraser et al., 2004; Konstantinidis and Tiedje, 2004, 2005; Markowitz et al., 2012; Ochman and Davalos, 2006;

Figure 3.2 Progress in genome sequencing 2007–2014. Tally of genome sequencing projects sorted by domain (*Bacteria*, *Archaea*, *Eukarya*, Virus; Metagenome). (Source: US DOE Joint Genome Institute. Genomes on-line database (GOLD). Web address: http://www.genomesonline.org/cgi-bin/GOLD/index.cgi.)

Syvanen, 2012; Wu et al., 2009; Zhou et al., 2004; Ozen et al., 2013). The sequence of an organism's genome reveals the blueprint of its current physiological capabilities and the integrated history of its heritage. In the last decade, genome-sequencing efforts have provided a remarkable amount of fundamental information about microbial life. The record of accrual of genomic information is shown in Figure 3.2. Since the first completed bacterial genome (*Haemophilus influenzae*) in 1995, advances in both DNA sequencing technology and supporting bioinformatic capabilities have spurred the genomic revolution of modern biology (details of sequencing technology appear in Section 6.10). Shown in Figure 3.2 is the 2014 tally of sequencing projects for individual cultures of *Bacteria*, *Archaea*, viruses and *Eukarya*, along with community metagenomics (the latter is discussed in Sections 6.9 and 6.10). As of early 2014, ~40,000 bacterial genomes, ~1000 archaeal genomes, and ~3000 eukaryotic microbial (fungi, protozoa, algae) genomes were either in completed or draft forms. Thousands more were in progress. Table 3.1 provides a representative list of genomes of prokaryotes isolated from ocean water, soils, sediments, sewage, or hot springs. These are cultured microorganisms (see Section 5.1) whose physiological properties make them valuable for understanding environmentally significant biogeochemical

Table 3.1

A sampling of completed prokaryotic genomes. (Modified from US Department of Environment Joint Genome Institute)

Organism	Genome size (Mb)	ORFs	Phenotype/habitat
Bacteria			
Acinetobacter sp. ADPI	3.59	3425	Aerobic chemoheterotroph and human pathogen/water, soil, human skin
Agrobacterium tumefaciens C58 Dupont	5.67	5467	Aerobic chemoheterotroph/soil, plant pathogen
Anabaena variabilis ATCC29413	7.10	5720	Oxygenic photosynthesis, nitrogen fixation/ water, soil
Bacillus cereus ATCC 14579	5.42	5397	Aerobic spore-forming heterotroph/soil
Bradyrhizobium japonicum USDA 110	9.10	8371	Aerobic heterotroph, nitrogen-fixing symbiont on soybean roots/soil
Buchnera aphidicola APS	0.655	609	Insect endosymbiont
Burkholderia xenovorans LB400	9.73	8784	Aerobic heterotroph, metabolizes polychlorinated biphenyl/soil
Candidatus Tremblaya princeps	0.139	110	Obligate endosymbiont of a citrus-feeding mealy bug; this bacterium harbors an endosymbiont
Carsonella rudii	0.160	182	Obligate endosymbiont of a psyllid, a plant sap-feeding insect
Clostridium acetobutylicum ATCC 824	4.13	3955	Obligate anaerobic spore-forming chemoorganotroph/soil
Dechloromonas aromatica RCB	4.50	4247	Facultative benzene degrader/water, soil
Deinococcus radiodurans R1	3.28	3239	Chemoheterotroph, aerobe, highly radiation resistant/soil
Desulfovibrio desulfuricans G20	3.73	3853	Strict anaerobe, chemoorganotroph using substrate as electron acceptors/sediment
Dehalococcoides ethenogenes strain 195 (now *D. mccartyi)*	1.47	1629	Strict anaerobe using chlorinated solvents as final electron acceptor/sewage, groundwater
Escherichia coli K12	4.64	4359	Facultative chemoheterotroph/human intestine
Escherichia coli O157:H7 EDL933	5.62	5622	Facultative chemoautotroph, human pathogen/intestine, food
Geobacter metallireducens GS-15	4.01	3587	Anaerobic chemoheterotroph using metal anions as electron acceptors/water, subsurface sediment
Mesotoga prima MesG1.Ag 4.2	2.97	2736	Strict anaerobe containing a reductive dehalogenase gene; isolated from polychlorinated biphenyl (PCB)-contaminated sediment
Methylococcus capsulatus Bath	3.30	3012	Aerobic methanotroph/soil water
Mycoplasma genitalium	0.580	516	Intracellular human parasite of urogenital tract

Table 3.1 *Continued*

Organism	Genome size (Mb)	ORFs	Phenotype/habitat
Nitrobacter winogradskyi Nb-255	3.40	3174	Facultative autotroph, CO_2 fixation, nitrite oxidizer/soil
Pelagibacter ubique (SARII) HTCC 1062	1.309	1394	Aerobic marine heterotroph/coastal temperate NE Pacific
Pelagibacter ubique (SAR 11) HIMB59	1.410	1532	Aerobic marine heterotroph/coastal tropical N Pacific
Polaromonas naphthalenivorans CJ2	5.34	5022	Chemoorgano- and lithotrophic aerobe/terrestrial sediment
Prochlorococcus marinus MIT 9312	1.70	1852	Photosynthetic bacterioplankton/ocean
Prochlorococcus marinus MIT 9313	2.41	2321	Photosynthetic bacterioplankton/ocean
Pseudomonas fluorescens Pf-5	7.07	6223	Aerobic chemoheterotroph/soil
Pseudomonas putida KT2440	6.18	5446	Aerobic chemoautotroph/soil
Rhodopseudomonas palustris CGA009	5.86	4897	Physiologically versatile-facultative photosynthetic organism/water, soil
Shewanella oneidensis MR-1	5.13	4601	Metabolically versatile chemoheterotroph, metal reduction/lake sediment
Silicibacter pomeroyi DSS-3	4.60	4314	Aerobic heterotroph important in the sulfur cycle/seawater
Sorangium cellulosum So ce 56	13.0	9700	Aerobic hterotroph with complex developmental life cycle (member of the myxobacteria)
Streptomyces avermitilis MA-4680	9.11	7759	Aerobic chemoheterotroph, filamentous spore former/soil
Synechococcus elongatus PCC 7942	2.74	2712	Photosynthetic bacterioplankton/ocean
Thermotoga maritima MSB8	1.86	1907	Hyperthermophilic anaerobe/geothermal marine sediment
Trichodesmium erythraeum ImS101	7.75	4494	Photosynthetic nitrogen-fixing filamentous cyanobacteria/ocean water
Archaea			
Archaeoglobus fulgidus DSM 4304	2.18	2519	Strict anaerobe, hyperthermophilic, sulfate reduction/hot springs
Cenarchaeum symbiosum A	2.0	2066	Aerobe, psychrophile, obligate symbiont within marine sponge
Halobacterium salinarum NRC-1	2.57	2726	Chemoorganotrophic aerobe/highly saline ponds and lakes
Haloferax volcanii DS2, ATCC 29605	4,0	4064	Chemoorganotrophic aerobe, mesophilic/salty shore of the Dead Sea
Methanopyrus kandleri AV19	1.69	1765	Strict anaerobe hyperthermophilic, methanogenesis/hot springs
Methanosarcina barkeri Fusaro	4.87	3854	Strict anaerobe, methanogenesis, cellulose metabolism/marine mud, sludge
Nanoarchaeum equitans Kin4-M	0.49	608	Intracellular parasite, anaerobic hyperthermophile/hot springs

Table 3.1 *Continued*

Organism	Genome size (Mb)	ORFs	Phenotype/habitat
Nitrosopulilus maritinus SCM1	1.64	1842	Aerobic, mesophile, autotrophic ammonia oxidizer/marine
Pyrococcus furiosus DSM 3638	1.90	2179	Strict anaerobe hyperthermophilic, radiation resistant, sulfur respiration/hot springs
Sulfolobus solfataricus P2	2.99	3141	Strict aerobe hyperthermophilic, acidophile, sulfur oxidizer/hot springs
Thermoplasma volcanium GSS1	1.58	1610	Facultative acidophilic thermophile/ hydrothermal vent

Mb, mega bases, 10^6 base pairs; ORF, open reading frame; Integrated Microbial Genomes website: http://img.jgi.doe.gov/cgi-bin/w/main.cgi.

processes such as the cycling of carbon, nitrogen, sulfur, metals, and organic environmental pollutants. Also represented in Table 3.1 are several well-characterized parasitic and pathogenic prokaryotes because these reveal several key features of prokaryotic biology. The US Department of Energy has sponsored a comprehensive effort to compile and analyze genomic information on *Bacteria* and *Archaea*. The result is a valuable and constantly expanding resource accessible via the world wide web, "Genomic Encyclopedia of *Bacteria* and *Archaea*" (GEBA; Wu et al., 2009; http://jgi.doe.gov/our-science/science-programs/microbial-genomics/phylogenetic-diversity/).

One lesson from the data in Table 3.1 is the rough correspondence between 10^3 base pairs (bp) of DNA and a single open reading frame (ORF) or gene. Although there is significant variation in size (number of amino acids) among proteins, they average somewhat more than 300 amino acids each. With three codons per amino acid, each ORF consists of about 10^3 bases. Bioinformatic software divides the long stretches of DNA bases into ORFs based on recognition of start and stop sites for protein translation on the encoded mRNA. A minimum ORF size of ~300 nucleotides is routinely assumed. Codon bias (preferential use in a given microbe of particular triplet base sequences in mRNA to encode a given amino acid) also helps define ORFs. It is important to realize that the initial judgments on ORFs are theoretical; their accuracy would ideally be confirmed subsequently by genetic and physiological tests.

Another lesson from the genome size comparison shown in Table 3.1 is that two strains of the same species can possess strikingly different genotypes and phenotypes. This is illustrated by the *Escherichia coli*, *Prochlorococcus*, and *Pelagibacter ubique* entries. Acquisition of DNA by an ancestor of *E. coli* strain K12 appears to have converted the benign

intestinal tract resident to an often lethal human pathogen (*E. coli* strain O157:H7; Table 3.1; see Chapter 5, Science and the citizen box). Extending the same theme, a recent broad comparison of the genomes of environmental and human-gut-associated *E. coli* indicates that the latter group reflects intestine-specific pressures manifest as enrichment of genes coding for the metabolism of *N*-acetylglucosamine, gluconate, and fucose (Luo et al., 2011). Similarly, genetic adaptations have been found within the genus *Prochlorococcus* (Table 3.1; an ecologically important photosynthetic marine bacterium): the strain with the larger genome has been shown to occupy a distinctive ecological niche characterized by low light intensity (Rocap et al., 2003). Furthermore, within the prominent marine heterotrophic *Pelagibacter* group, strain HTCC (Table 3.1) possesses a highly streamlined genome (lacking many genes common to other heterotrophs; Grote et al., 2012). In contrast, though also streamlined, *Pelagibacter* strain HIMB59 features 388 genes, absent from other *Pelagibacter* genomes, that confer traits ranging from glycolysis to phosphate utilization (Grote et al., 2012).

Another clear trend in the entries in Table 3.1 is the wide range in sizes of prokaryotic genomes. The smallest genomes are those of intracellular parasites: *Carsonella rudii* (featuring 182 ORFs encoded by a DNA sequence of 159,662 bp; Nakabachi et al., 2006), *Buchnera aphidicola* APS (featuring 609 ORFs encoded by a DNA sequence of 655,725 bp), *Mycoplasma genitalium* (featuring 516 ORFs encoded by a DNA sequence of 580,074 bp), *Nanoarchaeum equitans* Kin4-M (featuring 573 ORFs encoded by a DNA sequence of 490,885 bp), and *Candidatus* Tremblaya princeps (featuring 110 ORFs encoded by a DNA sequence of 138,931 bp). In contrast, among the largest prokaryotic genomes are free-living heterotrophic *Bacteria* from the soil habitat: *Bradyrhizobium japonicum* (featuring 8371 ORFs, encoded by a DNA sequence of 9,105,828 bp), *Burkholderia xenovorans* LB400 (featuring 8784 ORFs encoded by a DNA sequence of 9,703,676 bp), and *Streptomyces avermililis* (featuring 7759 ORFs encoded by a DNA sequence of 9,119,895 bp). After comparing the genomic composition of 115 prokaryotes, Konstantinidis and Tiedje (2004) argued persuasively for two hypotheses: (i) the small genomes of intracellular parasites are streamlined because they are relieved of selective pressures for maintaining elaborate metabolic pathways required for a free-living lifestyle and (ii) the large genomes of soil-dwelling microorganisms accommodate many genes (both regulatory and structural) that confer traits such as slow growth and exploitation of resources that are both diverse and scarce. Two additional features can contribute to large genome size in bacteria: "junk DNA" (noncoding DNA that fails to be translated into functional or regulatory proteins) and a complex development life cycle. *Sorangium cellulosum* features a huge genome (13,033,779 bp) probably because it is a soil-dwelling myxobacterium with an intricate social and developmental existence requiring a large number of sensory, biosynthetic, and structural

proteins. *S. cellulosum* also has an unusually high content (14.5%) of non-coding DNA (Schneiker et al., 2007).

Gene content within microbial genomes

After the bioinformatics software applies recognition algorithms that delineate individual genes (ORFs), then the ORFs are aligned with sequences of homologous genes of known function. These two steps allow amino acid similarities in proteins encoded by new and previously characterized genes to be computed so that the biochemical function of the new genes can be tentatively assigned. Several different levels of confidence for assigning gene function have been proposed (Kolker et al., 2005; Beckloff et al., 2012) and these vary, from high to low, depending upon the degree of similarity with known genes and proteins. In the earliest period of genomics (when DNA sequencing was expensive and laborious), annotation was carried out by teams of expert curators who manually confirmed ORFs and assigned function based on intimate knowledge of genes and experimental data from physiology and enzymology. Such curation efforts are still considered the "gold standards" in genome annotation (Beckloff et al., 2012). As more genomic data became available, especially due to next-generation sequencing technologies (see Section 6.10), computational algorithms embedded in gene-prediction computer programs (such as "Glimmer" have paved the way to current genome annotation "pipelines" (see Box 6.6). Sequence comparisons between new genes and previously annotated genes are the basis for assigning function to newly sequenced genes. If the degree of match is high and the reference gene was accurately named, then the new annotation is extremely likely to be accurate. However, if an error was made in naming the original reference gene, then errors in annotation occur and can be propagated rapidly throughout the sequence databases. Thus, care must be exercised when interpreting modern genomic annotations; misannotation is not uncommon (Beckloff et al., 2012).

The template used by the US DOE Joint Genome Institute for categorizing and organizing genome sequence data is shown in Table 3.2. There are 23 functional categories of expected genes, known as COGs (clusters of orthologous groups of proteins), which span three well-established necessities of cellular life: "information storage and processing", "cellular processes", and "metabolism". The remainder of genes in a genome fall into the "poorly characterized" category ("function unknown" and "general function prediction only"). Results of COG analysis for the 6.18 Mb genome (5481 ORFs) of *Pseudomonas putida* KT2440 are presented in Table 3.2. Note the high apportionment of this soil bacterium's genome to energy production (5.4%) and amino acid uptake and metabolism (9.5%); this issue will be revisited in Section 3.3.

Table 3.2

Framework for categorizing and organizing open reading frames (ORFs) found during genome-sequencing projects. Twenty-five gene categories (cluster of orthologous groups of proteins: COGs) and their respective contributions to the sequenced genome of *Pseudomonas putida* KT2240 are listed. (From US Department of Environment Joint Genome Institute)

Functional group of genes (COG)	Example genome (*Pseudomonas putida* KT2440): number of genes in each functional group (% of total)
Information storage and processing	
J Translation, ribosomal structure and biogenesis	187 (3.4)
A RNA processing and modification	3 (0.05)
K Transcription	440 (8.1)
L DNA replication, recombination and repair	221 (4.0)
B Chromatin structure and dynamics	2 (0.04)
Cellular processes	
D Cell cycle control, cell division and chromosome partitioning	42 (0.77)
Y Nuclear structure	–
V Defense mechanisms	63 (1.2)
T Signal transduction mechanisms	330 (6.1)
M Cell wall/membrane/envelope biogenesis	266 (4.9)
N Cell motility	127 (2.3)
Z Cytoskeleton	–
W Extracellular structures	–
U Intracellular trafficking, secretion, and vesicular transport	123 (2.3)
O Posttranslational modification, protein turnover, chaperones	174 (3.2)
Metabolism	
C Energy production and conversion	293 (5.4)
G Carbohydrate transport and metabolism	228 (4.2)
E Amino acid transport and metabolism	516 (9.5)
F Nucleotide transport and metabolism	91 (1.7)
H Coenzyme transport and metabolism	186 (3.4)
I Lipid transport and metabolism	181 (3.3)
P Inorganic ion transport and metabolism	275 (5.1)
Q Secondary metabolites biosynthesis, transport and catabolism	128 (2.3)
Poorly characterized	
R General function prediction only	564 (10.4)
S Function unknown	1014 (18.6)

The "poorly characterized" or "unknown hypothetical" category of genes is a barometer for the completeness of our understanding of prokaryotic genomes and (by inference) prokaryotic biology. The proportion of unknown hypothetical genes in all genome-sequencing projects hovers at roughly one-third (e.g., Kolker et al., 2005; Beckloff et al., 2012). This proportion of unknown hypothetic genes even applies to the thoroughly studied bacterium, *E. coli*. While it is possible that poorly characterized genes have no function (they may be noncoding or "junk" DNA), it is equally plausible that these genes are blatant reminders of how little we truly understand about real-world ecological pressures and the physiological processes expressed by microorganisms in their native habitats. *Because laboratory cultivation of microorganisms likely fails to mimic the physiological and ecological conditions experienced by microorganisms in field settings, many genetic traits may go unexpressed by the organism and, hence, be unobserved by the microbiologist*. Understanding the function of unknown hypothetical genes represents one of the major frontiers in biology. The lack of knowledge of a significant proportion of each microbial genome limits our ability to advance biology to a more predictive science (Kolker et al., 2005; Hanson et al., 2009). Thus, although genomics has provided genetic blueprints for individual microorganisms, a substantial proportion of the blueprints remains "illegible" and mysterious.

Integrating genome data: gene functionality and phylogenetic diversity

Even with roughly one-third of each microbial genome obscured (see above) the insights from the remaining (legible) portion of each genome are tremendously powerful. For each sequenced genome, a template for cell function is used to organize the genes from the 23 functional gene categories of Table 3.2. Figure 3.3 shows the metabolic template for a model cell. The generalized conceptual cell model exhibits an interior (cytoplasm) and an exterior bounded by the cytoplasmic membrane and the cell wall. The periphery of the cell is lined with membrane-bound transport proteins that regulate cytoplasmic composition by acting on inorganic cations, inorganic anions, carbohydrates, amino acids, peptides, purines, pyrimidines, other nitrogenous compounds, carboxylates, aromatic compounds, other carbon compounds, and water. Many of the transport mechanisms are energy-coupled (requiring ATP) and show a recognizable ATP binding cassette (ABC) motif. Channel proteins transport materials both into and out of the cell, as do P-type ATPase transport systems for uptake and efflux.

The interior of the model cell (Figure 3.3) contains a template for organizing products (proteins) of recognized genes involved in metabolism. Similar templates apply to genes functioning in information storage and other cellular processing. Structural and regulatory genes encoding metabolic processes (respiration, the tricarboxylic acid cycle, other biochemical pathways, etc.) appear as networks within the cell's interior. Figure 3.4 extends

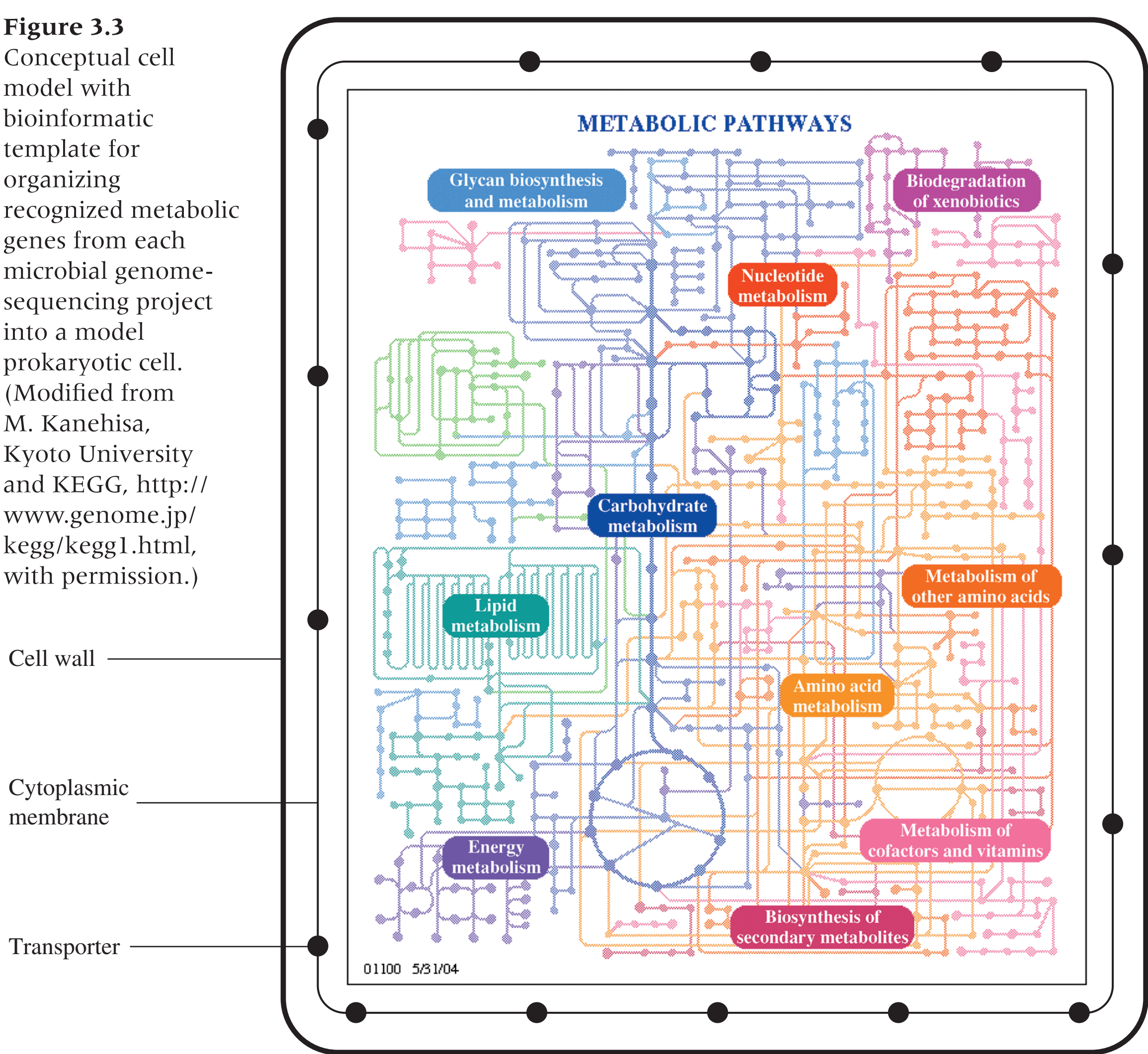
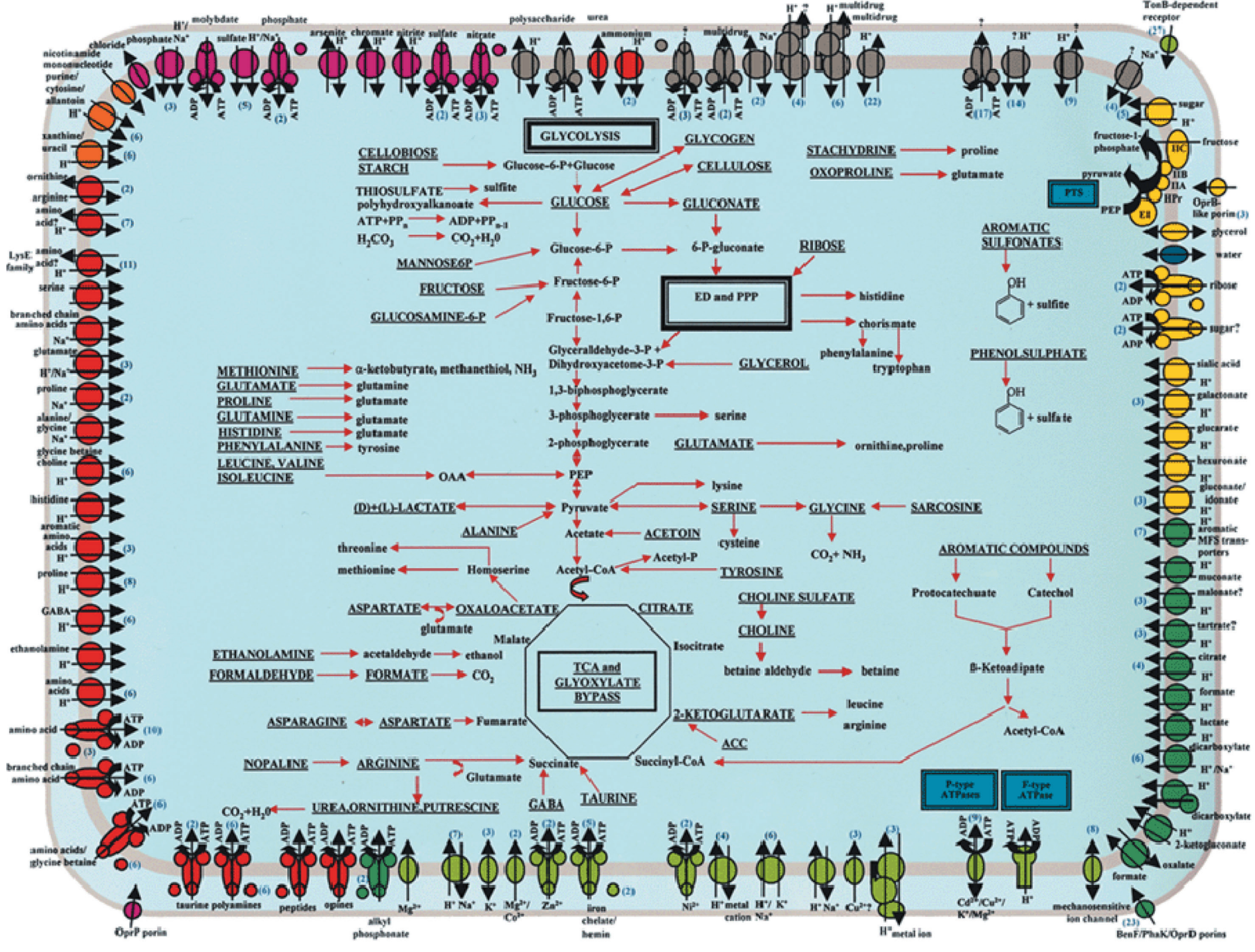


Figure 3.3 Conceptual cell model with bioinformatic template for organizing recognized metabolic genes from each microbial genome-sequencing project into a model prokaryotic cell. (Modified from M. Kanehisa, Kyoto University and KEGG, http://www.genome.jp/kegg/kegg1.html, with permission.)

Figure 3.4 (*opposite*) A modeled genome for *Pseudomonas putida* KT2440. Shown is an overview of metabolism and transport based on the 6.18 Mb completed genome. Predicted pathways for energy production and metabolism of organic compounds are shown. Predicted transporters are grouped by substrate specificity: inorganic cations (light green), inorganic anions (pink), carbohydrates (yellow), amino acids, peptides, amines, purines, pyrimidines, and other nitrogenous compounds (red), carbohydrates, aromatic compounds, and other carbon sources (dark green), water (blue), drug efflux and other (dark gray). Question marks indicate uncertainty about the substrate transported. Export or import of solutes is designated by the direction of the arrow through the transporter. The energy-coupling mechanisms of the transporters are also shown: solutes transported by channel proteins are shown with a double-headed arrow; secondary transporters are shown with two arrowed lines, indicating both the solute and the coupling ion; ATP-driven transporters are indicated by the ATP hydrolysis reaction; transporters with an unknown energy-coupling mechanism are shown with only a single arrow. The P-type ATPases are shown with a double-headed arrow to indicate that they include both uptake and efflux systems. Where multiple homologous transporters with similar predicted substrate exist, the number of that type of transporter is indicated in parentheses. The outer and inner membrane are sketched in gray, the periplasmic space is indicated in light turquoise, and the cytosol in turquoise. (From Nelson, K.E., C. Weinel, I.T. Paulsen, et al., 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:799–808. With permission from Blackwell Publishing, Oxford, UK.)



GLYCOLYSIS
CELLOBIOSE
STARCH
Glucose-6-P+Glucose
THIOSULFATE
sulfite
polyhydroxyalkanoate
GLUCOSE
GLYCOGEN
CELLULOSE
GLUCONATE
Glucose-6-P
6-P-gluconate
RIBOSE
MANNOSE6P
FRUCTOSE
Fructose-6-P
ED and PPP
GLUCOSAMINE-6-P
Fructose-1,6-P
Glyceraldehyde-3-P +
Dihydroxyacetone-3-P
GLYCEROL
histidine
chorismate
phenylalanine
tryptophan
METHIONINE
GLUTAMATE
PROLINE
GLUTAMINE
HISTIDINE
PHENYLALANINE
LEUCINE, VALINE
ISOLEUCINE
glutamine
glutamate
tyrosine
1,3-biphosphoglycerate
3-phosphoglycerate
serine
2-phosphoglycerate
ornithine,proline
OAA
PEP
lysine
(D)+(L)-LACTATE
Pyruvate
SERINE
GLYCINE
SARCOSINE
ALANINE
Acetate
ACETOIN
cysteine
threonine
methionine
Homoserine
Acetyl-CoA
Acetyl-P
TYROSINE
ASPARTATE
OXALOACETATE
CITRATE
glutamate
Malate
Isocitrate
ETHANOLAMINE
acetaldehyde
ethanol
FORMALDEHYDE
FORMATE
TCA and
GLYOXYLATE
BYPASS
CHOLINE SULFATE
CHOLINE
betaine aldehyde
betaine
ASPARAGINE
Fumarate
2-KETOGLUTARATE
leucine
arginine
ACC
NOPALINE
ARGININE
Succinate
Succinyl-CoA
Glutamate
GABA
TAURINE
UREA,ORNITHINE,PUTRESCINE
STACHYDRINE
proline
OXOPROLINE
PTS
AROMATIC
SULFONATES
+ sulfite
PHENOLSULPHATE
+ sulfate
AROMATIC COMPOUNDS
Protocatechuate
Catechol
ß-Ketoadipate
Acetyl-CoA
P-type
ATPases
F-type
ATPase
TonB-dependent
receptor
sugar
fructose
fructose-1-
phosphate
pyruvate
PEP
OprB-
like porin
glycerol
water
ribose
sugar?
sialic acid
galactonate
glucarate
hexuronate
gluconate/
idonate
aromatic
MFS trans-
porters
muconate
malonate?
tartrate?
citrate
formate
lactate
dicarboxylate
2-ketogluconate
oxalate
mechanosensitive
ion channel
BenF/PhaK/OprD porins
OprP porin
taurine
polyamines
peptides
opines
alkyl
phosphonate
iron
chelate/
hemin
metal ion
molybdate
phosphate
sulfate
arsenite
chromate
nitrite
nitrate
polysaccharide
urea
ammonium
multidrug
chloride
nicotinamide
mononucleotide
purine/
cytosine/
allantoin
xanthine/
uracil
ornithine
arginine
amino
acid?
LysE
family
serine
branched chain
amino acids
glycine betaine
choline
histidine
aromatic
amino
acids
proline
GABA
ethanolamine
amino acid
branched chain
amino acid
amino acids/
glycine betaine

the general model to a portion of the functional gene data presented in Table 3.2. Shown in Figure 3.4 is an overview of the integrated genetic information for metabolism and transport in *Pseudomonas putida* KT2440. Analysis of this 6.18 Mb genome provides a paradigm for the physiological and ecological function of pseudomonads – a broad class of opportunistic and versatile bacteria that are widespread in terrestrial and aquatic environments. The genome data are the basis for a wealth of hypotheses about genetic determinants for transporters, oxygenase enzymes, electron transport chains, sulfur metabolism proteins, and microbial mechanisms for protection from toxic pollutants and their metabolites (Nelson et al., 2002).

Genomic representations such as that shown in Figure 3.4 provide unrivaled and comprehensive views of what microorganisms are, what they can do, and what each of their evolutionary histories have been. Genome-sequencing projects (Table 3.1) are not an end in themselves. Rather, such blueprints are the gateway to a variety of tools that extend and refine our understanding of prokaryotic biology. To test hypotheses that arise from genome-sequencing data, other essential tools include biochemical assays, physiological assays, transcriptome assays, proteome assays, site-directed mutagenesis creating knockout and other mutations, genetic complementation, computational modeling, systems biology (see Section 9.1), and tests of ecological relevance (the latter are described in Sections 6.11 and 9.2).

Genomics also plays a huge role in developing our understanding of the evolution and diversity of life on Earth (Wu et al., 2009; Hedlund et al., 2014; Rinke et al., 2013; Spang et al., 2013). Genetic innovations (e.g., new gene variants in new combinations) across *Bacteria*, *Archaea*, and *Eukarya* (See Section 5.5) are established in response to our planet's heterogeneity of resources and selective pressures. Molecular phylogeny based on small subunit rRNA genes was the basis for the current three-domain tree of life, but a tree of life based on a single gene (encoding 16S and 18S rRNA) often features ambiguities. Greater phylogenetic resolution and robustness can be achieved using alignments of multiple, universally distributed functional genes, such as those involved in transcription, translation, DNA repair, stress response, etc. Clearly, access to complete (or draft) genomes is required for constructing phylogenetic trees based upon multiple genes. Two recent technological developments have enabled bioinformatic assembly of draft genomes from uncultivated microorganisms. These techniques are: (i) single-cell genomics and (ii) next-generation-sequencing-based metagenomics (see Sections 6.9 and 6.10). Just as sequences of environmentally derived small-subunit rRNA genes can be merged into phylogenetic trees of cultivated microorganisms, so also constellations of multiple genes from genomes of uncultivated microorganisms can be merged with those of cultivated microorganisms – thus extending, refining, and integrating efforts that explore phylogenic

diversity (Hedlund et al., 2014; Rinke et al., 2013; Spang et al., 2013; see Section 5.7).

3.3 FUNDAMENTALS OF NUTRITION: CARBON- AND ENERGY-SOURCE UTILIZATION PROVIDE A FOUNDATION FOR PHYSIOLOGICAL ECOLOGY

All forms of life (from prokaryotes to humans) need to make a living. To achieve this, we all require at least two physiological resources: (i) an energy source for generating ATP and (ii) a carbon source for assembling the cellular building blocks during maintenance of existing cells and/or creation of new cells (growth). No life form can exist without abilities to successfully exploit well-defined energy and carbon sources. Therefore, when microbial physiologists and microbial ecologists approach a new organism or a new habitat (from deep subsurface sediments, to ocean water, to insect guts, to Mars), the first questions to ask are: "What drives metabolism here? What are the energy and carbon sources?" The answers provide fundamental nutritional bases for subsequent hypotheses and inquiries about past and ongoing biogeochemical and ecological processes.

Table 3.3 provides a useful framework for classifying the nutritional needs of individual microorganism and ecosystems alike. The matrix shows the energy source along the top and carbon source along the left side. Energy sources fall into two main categories: chemical and light. The chemical sources of energy are further divided into two: inorganic (such as hydrogen gas, ammonia, methane, and elemental sulfur) or organic compounds (generally containing multiple C–C bonds). The carbon sources also fall into two categories: gaseous CO_2 or fixed organic carbon. The matrix in Table 3.3 provides a means for nutritionally classifying virtually all forms of life on Earth into five groups. The two that are perhaps easiest to recognize are plants and animals (including humans). Plants are *photosynthetic autotrophs* – deriving energy from light and carbon from gaseous CO_2. Humans, fungi, and microorganisms that cycle plant-derived (and other) organic substrates are *chemosynthetic organoheterotrophs*. Microorganisms that oxidize inorganic compounds (e.g., hydrogen gas, ammonia, elemental sulfur) for energy and assimilate CO_2 into their biomass are *chemosynthetic lithoautotrophs*. Microorganisms that use light as an energy source and assimilate fixed carbon into their biomass are *photosynthetic heterotrophs* (this lifestyle is exclusively prokaryotic and rare). Microorganisms that use inorganic compounds as energy sources and assimilate fixed carbon into their biomass are *chemosynthetic lithoheterotrophs* (this lifestyle is exclusively prokaryotic and extremely rare). It should be recognized that the boundaries between the five lifestyle categories in Table 3.3 may sometimes be blurred.

Table 3.3

Physiological classification of life forms based on energy source and carbon source. The five categories assist in understanding both individual microorganisms and biogeochemical systems

Carbon source	Energy source		
	Chemical, organic	**Chemical, inorganic**	**Light**
Fixed organic	**Chemosynthetic organoheterotroph** (Example: humans, fungi, *Pseudomonas*)	**Chemosynthetic lithoheterotroph** (Example: *Beggiatoa* sp.)	**Photosynthetic heterotroph** (Example: purple and green bacteria; *Rhodospirillum*)
Gaseous CO_2		**Chemosynthetic lithoautotroph** (Example: ammonia-, hydrogen-, and sulfur-oxidizing bacteria; *Nitrosomonas, Aquifex*)	**Photosynthetic autotroph** (Example: plants, algae, *Prochlorococcus*)

Terminology:

- Autotroph: carbon from CO_2 fixation
- Heterotroph: carbon assimilated from (fixed) organic compounds
- Photosynthetic: energy from light
- Chemosynthetic: energy from oxidizing reduced chemicals
- Chemolitho: energy from oxidizing inorganic reduced chemicals
- Chemoorgano: energy from oxidizing organic reduced chemicals.

For instance, some microorganisms are genetically and physiologically versatile – adopting a heterotrophic lifestyle when fixed organic carbon is available, then resorting to CO_2 fixation in the absence of organic carbon. Regardless of occasional minor ambiguities, the above-described nutritional concepts and terminology are insightful and will be used throughout this text.

Figure 3.5 shows a bar graph revealing general trends in the proportions of genes represented in genomes in four major prokaryotic lifestyles. COG analysis (see Section 3.2) of three organisms in each nutritional class suggests that the transport and metabolism of both amino acid and carbohydrates have been emphasized during the evolution of chemosynthetic organoheterotrophs (especially soil-dwelling bacteria). For photosynthetic heterotrophs, a relatively large portion of the genome has been relegated to energy production and conversion. Further, there seems to be a trend in photoautotroph evolution that de-emphasizes a need for inorganic ion transport and metabolism. The trends suggested in Figure 3.5 may change as additional genomes are completed and/or the members of each nutritional class are expanded. Nonetheless, the notion of mechanistic links between evolutionary pressures and gene content is a compelling one that is likely to be extensively pursued in the future.

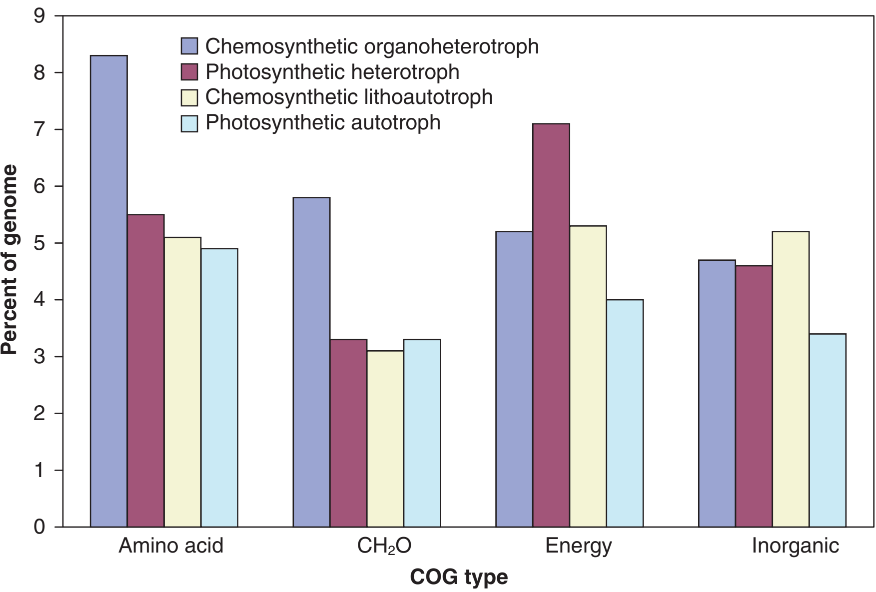


Figure 3.5 Apportionment of metabolic genes (as percent of total genome) among representatives of the four major nutritional categories in prokaryotes: chemosynthetic organoheterotrophs (purple), photosynthetic heterorophs (red), chemosynthetic lithoautotrophs (green), and photosynthetic autotrophs (blue). Bar graph shows the percent of the genome devoted to amino acid transport + metabolism, carbohydrate (CH_2O) transport + metabolism, energy production + conversion, and inorganic ion transport + metabolism. Data are based on COG analysis of 12 genomes. Representatives of chemosynthetic organoheterotrophs were *Pseudomonas putida* KT2440, *Bacillus cereus* ATCC 14579, and *E. coli* K12. Representatives of photosynthetic heterotrophs were *Chlorobium tepidum* TLS, *Chlorochromatium aggregatum*, and *Chlorobium lumicola* CSMZ 245 (T). Representatives of chemosynthetic lithoautotrophs were *Nitrosomonas europea* ATCC 19718, *Nitrobacter winogradskyi* Nb-255, and *Thiobacillus denitrificans* sp. ATCC 25259. Representatives of photosynthetic autotrophs were *Prochlorocuccus marinus* MIT 9313, *Trichodesmium erythraeum* IMS101, and *Anabaena* sp. PCC7120. (Data from US Department of Energy Joint Genome Institute.)

3.4 SELECTIVE PRESSURES: ECOSYSTEM NUTRIENT FLUXES REGULATE THE PHYSIOLOGICAL STATUS AND COMPOSITION OF MICROBIAL COMMUNITIES

In keeping with the concepts displayed in Figure 3.1, it is the dialog between Earth's habitats and their microbial inhabitants that has directed the course of prokaryotic evolution and the development of microbial diversity. Thus, knowing the resources, the nutritional status, and the geochemical composition of our biosphere can help us understand

pressures for genetic selection and adaptation. Consider the following questions.

- **What was it like to be an early microorganism in Earth's primordial seas?**
- **What physiological and ecological pressures do contemporary microorganisms face in aquatic and terrestrial habitats?**
- **What are their carbon and energy sources?**
- **How important is nutrient uptake? Starvation? Competition? Predation? Parasitism?**
- **To what degrees do issues such as acid stress, oxidative stress, temperature extremes, desiccation, or UV radiation influence physiology?**

The unifying answer to questions such as those above is based on Darwinian evolutionary theory: *the prime directive for prokaryotic life is survival, cell maintenance, ATP generation, and growth*. The answer applies to ancient and modern microorganisms under all environmental conditions. Without growth, replication of genes cannot occur and evolution is thwarted.

- **But what is the nature of microbial growth in real-world habitats (waters, sediments, soils)?**
- **Is it rapid or slow? Constant or sporadic?**
- **And what habitat conditions control microbial growth?**

To emphasize the rarity of extended periods of rapid microbial growth in natural habitats, Stanier et al. (1986) developed the following scenario:

> In 48 h, a single bacterium (weighing 10^{-12} g) exponentially doubling its biomass every 20 min would produce progeny weighing 2.2×10^{31} g, or roughly 4000 times the weight of the Earth!

Clearly then, extended rapid exponential growth is not the status quo for microorganisms in nature. The alternatives to rapid exponential growth are: (i) sporadic rapid growth (rapid growth when resources are available followed by a quiescent stage); (ii) slow growth; (iii) sporadic slow growth; and/or (iv) dormancy. Figure 3.6 displays the key types of physiological and ecological pressures that confront microorganisms in nature. The illustration depicts a planktonic microorganism in the water column of a lake, sea, or ocean. The forces of nature confronted by this cell and fellow members of its population are complex. Physical, biological, and chemical properties of habitats are dynamic – varying in space and in time. The cell has three potential fates resulting from its interactions with its habitat: (i) ecological success; (ii) ecological failure; or (iii) survival/maintenance.

As shown in Figure 3.6, ecological success would be manifest as growth and increased population size. Ecological success occurs when energy and carbon courses are exploited, other nutrients are taken up, and potentially adverse physiological and ecological obstacles are overcome. Ecological failure is manifest by population decline, death, and/or elimination from the habitat. Ecological failure occurs when detrimental environmental, physiological, and/or ecological factors (starvation, competition, predation, parasitism, washout, oxidative stress, toxins, UV light, etc.) overwhelm a population's ability to survive and grow. The survival/maintenance state is the "neutral, wait and see" middle ground between ecological failure and success. The survival/maintenance state relies upon a suspension of rapid growth – substituting a status of sporadic slow growth or a quiescent, dormant state in which cellular activities have drastically slowed or stopped (for further discussion of dormancy, see Section 3.5).

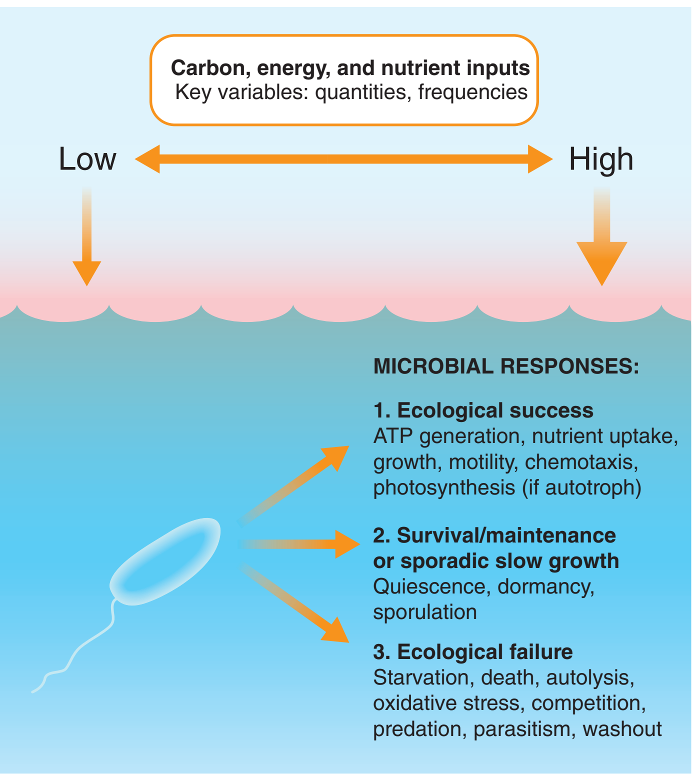


Figure 3.6 Selective pressures influencing a typical microbial cell dwelling in the water column of a lake, sea, or ocean. Three outcomes of cell responses are ecological success, ecological failure, or survival/maintenance.

In describing microbial habitats, a crucial feature of Figure 3.6 is the input of carbon and energy (and other nutrient) sources. These input fluxes provide adaptive pressures and exert major influences on system productivity, standing biomass, metabolic rates, physiological status, and the composition of microbial communities. Poindexter (1981) suggested that it is appropriate to define a given habitat in terms of the average flux of nutrients. Nutrient-rich lakes and areas of ocean shores are *eutrophic* when they have a flux of organic carbon of at least 5 mg C/L/day (Poindexter, 1981). In contrast, a nutrient-poor lake and areas of the open ocean offering *oligotrophic conditions* are defined as having a flux of organic carbon that does not exceed 0.1 mg C/L/day (see Box 3.1 for definitions of terms pertinent to nutrients, habitats, and microorganisms).

Types of microorganisms adapted to ecological success in the above two habitat categories have been termed eutrophs (alternatively copiotrophs; Poindexter, 1981) and oligotrophs, respectively. According to Poindexter (1981), oligotrophic bacteria are conceived to be those whose survival in

Box 3.1

Terms, concepts, and definitions relevant to the nutritional status of habitats and their microbial inhabitants

Term	Definition/use
Oligotrophic	Adjective that describes a habitat or microorganism. Low nutrient status. For heterotrophs in lakes, the carbon (C) flux is <0.1 mg C/L/day
Oligotroph and olilgocarbophile	Class of microorganism adapted to life in nutrient-poor, oligotrophic habitats
Eutrophic	Adjective that describes a habitat or microorganism. High nutrient status. For heterotrophs in lakes, the C flux is >5 mg C/L/day
Eutroph and copiotroph	Class of microorganism adapted to life in nutrient-rich, eutrophic habitats
r-selection, *r*-selected	Ecological terms referring to lifestyle strategy that features brief bursts of rapid growth in response to sporadic nutrient input
K-selection, *K*-selected	Ecological terms referring to lifestyle strategy that features slow constant growth
Maintenance energy	Nongrowth-related cellular energy demand used: to maintain intracellular pH and potential across the cytoplasmic membrane; for osmotic regulation; to transport solutes; to resynthesize macromolecules; for motility; and to counteract energy dissipation by proton leakage across membranes and by ATP hydrolysis
Stenoheterotrophic	Ecological term referring to a habitat with a narrow fluctuation in nutrient status (Horowitz et al., 1983)
Euryheterotrophic	Ecological term referring to a habitat with wide fluctuations in nutrient status
Dormancy	Quiescent, resting state assumed by vegetative cells, especially those unable to develop specialized resting stages. In the dormant state, energy expenditure counteracts macromolecular damage (depurination of DNA, racemization of amino acids), and maintenance of chemiosmotic ion gradients across membranes (Morita, 2000; Price and Sowers, 2004; Hoehler and Jorgensen, 2013)
Sporulation	A complex series of developmental steps in many prokaryotes and fungi that leads to the formation of a resistant resting structure, the spore. Spore formation is typically triggered by nutrient deprivation

nature depends on their ability to multiply in habitats of low nutrient flux. Proteins involved in membrane transport, substrate binding, and catalysis should be distinctive for oligotrophs, when compared to proteins that in eutrophic organisms operate at substrate concentrations at least 50-fold higher. Schut et al. (1997) have suggested that the "oligotrophic way of life" is a widespread transient physiological characteristic – especially of marine bacteria. A recent genomic survey of 137 ocean-plankton prokaryotes

supports the theory that slow growth and survival under energy-limited conditions is crucial for many marine microbes (Yooseph et al., 2010). Moreover, Carini et al. (2013) have used the broadly occurring marine heterotroph, *Pelagibacter ubique*, as a physiological and genomic model, to explore the genetic and ecological basis for oligotrophy.

The frequency at which pulses of nutrients are delivered to a given habitat (Figure 3.6) is likely to be as influential as the sizes of a given pulse. Some habitats (especially guts of carnivorous animals, soil litter layers beneath vegetation, and even the ocean floor which occasionally receives whale carcasses; Smith and Baco, 2003) can experience long periods with little or no nutrient inputs. These can be followed by a substantive input pulse. The frequency and quantities of nutrients delivered to waters, sediments, and soils has obvious implications for adaptive pressures of the microbial inhabitants and their chances of ecological success. For example, a “feast and famine” existence is very different from one that has low but constant nutrient inputs.

In recognition of the dynamic and widely varying nutritional characteristic of aquatic and terrestrial habitats (and their respective selective pressures), ecologists have developed the concept of *r*-selected and *K*-selected growth strategies (Box 3.1). The *r*-selected species are ones adapted to high rates of reproduction – they exploit nutrient inputs rapidly, exhibiting high rates of growth in an uncrowded habitat. In contrast, *K*-selected species are adapted to conserve resources – they exhibit slow, constant growth rates appropriate for habitats featuring crowded, high-density populations (Andrews and Harris, 1986; Atlas and Bartha, 1998). Using a foot-race metaphor for microbial growth strategies, *r*-selected species are good sprinters, while *K*-selected species are slow, steady endurance runners.

As used above, the terms oligotrophic and eutrophic apply to organic carbon-dependent chemoorganoheterotrophic nutrition. Table 3.4 extends the approach of habitat classification to two additional major physiological types of microorganisms: photoautotophs and chemolithotrophs. Each habitat type listed in Table 3.4 (ocean water, lake water, sediment, soil, and subsurface sediment) has its own set of physical, chemical, and biological characteristics that govern, enhance, and/or constrain microbial activity. For phototrophic life, clearly diurnal variations in sunlight represent a major habitat-imposed constraint. However, minerals may also govern the growth of photosynthetic microorganisms (see Section 7.1) – the open ocean is typically limited by iron availability while freshwaters are often limited by phosphorus. Regarding chemolithotrophic life, the fluxes and types of energy-limiting, reduced inorganic compounds vary with habitat types. Typically systems at the biosphere–geosphere boundary are dependent upon fluxes of volcanic or geothermal resources (especially H_2, CH_4, H_2S), while soil and freshwater sediment-type habitats rely on inorganic inputs (such as H_2, NH_3, and CH_4) produced by the processing of deceased biomass by anaerobic microbial food chains (see Sections 3.10 and 7.3).

Table 3.4
Predominant ecological limitations for energy and growth for three physiological classes of microorganisms in a variety of habitats

	Habitat characteristics and nutrient limitations faced by three physiological classes of microorganisms		
Habitat type	**Photoautotroph**	**Chemolithotroph**	**Chemoorganoheterotroph**
Ocean water	Daily light cycle, light penetration depth; scarce iron	Flux of reduced inorganic compounds, especially NH_3, H_2S, H_2, or CH_4 from nutrient turnover and hydrothermal vents	Carbon flux from phototrophs, dead biomass, and influent waters
Lake water	Daily light cycle, light penetration depth; scarce phosphorus	Flux of reduced inorganic materials, especially NH_3, H_2, and CH_4 from nutrient turnover	Carbon flux from phototrophs, dead biomass and influent waters
Sediment (freshwater and oceanic)	Daily light cycle, light penetration depth	Flux of reduced inorganic materials, especially NH_3 and H_2 from nutrient turnover or H_2, H_2S, or CH_4 from hydrothermal vents	Flux of organic carbon from phototrophs and dead biomass; flux of final electron acceptors to carbon-rich anaerobic strata
Soil	Daily light cycle, light penetration depth	Flux of reduced gaseous substrates, especially methane from nutrient turnover by anaerobes	Slow turnover of soil humus, dead biomass, plant root exudates; leaf fall from vegetation
Subsurface sediment	No light	Flux of reduced inorganic materials, especially H_2 and CH_4 from geothermal origin	Carbon flux from nutrient turnover

3.5 CELLULAR RESPONSES TO STARVATION: RESTING STAGES, ENVIRONMENTAL SENSING CIRCUITS, GENE REGULATION, DORMANCY, AND SLOW GROWTH

The life cycle of some specialized prokaryotes, fungi, and protozoa includes a resistant, quiescent stage, variously termed endospore, myxospore, cyst, conidium, etc. Such developmental stages in microbial life cycles are triggered by environmental cues, especially starvation, received by the microorganism. The resulting resting stages typically surround vital cytoplasmic constituents with a thick-walled structure that confers resistance not only to starvation but also to extreme environmental conditions ranging from heat, to desiccation, to acidity, to γ-irradiation, to salinity, to UV light. In many prokaryotes, the ultrastructure, physiology, and genetics of endospore formation have been well characterized (Box 3.2; e.g., Nicholson et al., 2000; Hilbert and Piggot, 2004; McKenney et al., 2013).

Box 3.2

Stages of *Bacillus* endospore formation and an electron micrograph of an endospore of *Sporosarcina ureae*

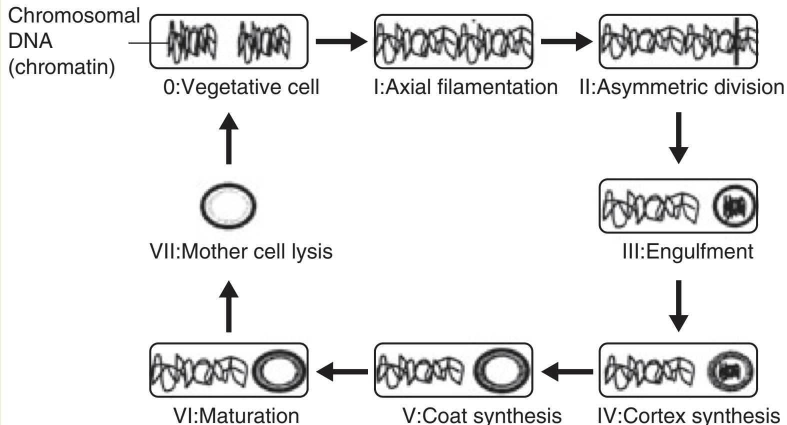


Figure 1 Schematic representation of the stages of spore formation. A vegetatively growing cell is defined as stage 0. It is shown as having completed DNA replication and containing two complete chromosomes (represented as disordered lines within the cells), although replication is not completed at the start of spore formation. Formation of an axial filament of chromatin, where both chromosomes (or a partially replicated chromosome) form a continuous structure that stretches across the long axis of the cell, is defined as stage I. Asymmetric division occurs at stage II, dividing the cell into the larger mother cell and smaller prespore; for clarity, the septum is indicated as a single line. At the time of division, only approximately 30% of a chromosome is trapped in the prespore, but the DNA translocase SpoIIIE will rapidly pump in the remaining 70%. Stage III is defined as completion of engulfment, and the prespore now exists as a free-floating protoplast within the mother cell enveloped by two membranes, represented by a single ellipse. Synthesis of the primordial germ cell wall and cortex, a distinctive form of peptidoglycan, between the membranes surrounding the prespore is defined as stage IV and is represented as thickening and graying of the ellipse. Deposition of the spore coat, protective layers of proteins around the prespore, is defined as stage V. The coat is represented as the black layer surrounding the engulfed prespore. Coincident with coat and cortex formation, the engulfed prespore is dehydrated, giving it a phase-bright appearance in a microscope, represented here as a light gray shading. Stage VI is maturation, when the spore acquires its full resistance properties, although no obvious morphological changes occur. Stage VII represents lysis of the mother cell, which releases the mature spore into the environment. (From Hilbert, D.W. and P.J. Piggot, 2004. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiol. Molec. Biol. Rev*. **68**:234–263. With permission from the American Society for Microbiology, Washington, DC.)

Box 3.2 *Continued*

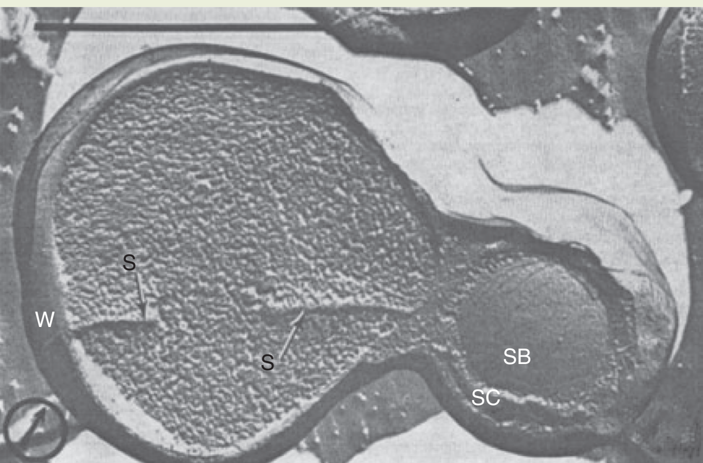


Figure 2 A freeze-etch prepared electron micrograph of an endosphere within an *Sporosarcina ureae* cell. SB, spore body; SC, spore coat; S, septa; W, cell wall. Scale bar, 0.5 µm. (From Holt, S.C. and E.R. Leadbetter, 1969. Comparative ultrastucture of selected aeorobic spore-forming bacteria: a freeze-etching study. *Bacteriol. Rev*. **33**:346–378. With permission from the American Society for Microbiology, Washington. DC.)

Specialized morphological adaptations to starvation, however, are not the rule in the microbial world. In fact, Hoehler and Jorgensen (2013) have stated that in some habitats (such as extremely stable, low-nutrient geologic formations) spore formation is disadvantageous to microbial populations because of the energetic costs associated with forming spore structures. Recent studies have examined the physiological and genetic responses of microorganisms to starvation using pure cultures (such as *E. coli*) that have exhausted nutrient supplies in laboratory growth media. In these model systems, genomic and biochemical assays have been applied to prokaryotes experiencing oxidative stress, senescence, programmed cell death, arrested growth, and mutations in long-term stationary phase culture (e.g., Nystrom, 2004; Finkle, 2006; Hoehler and Jorgensen, 2013). Results have reinforced the view that responsiveness to environmental conditions, especially those that cause physiological stress, is one of the hallmarks of prokaryotic behavior. Information about the chemical, physical, and nutritional status of a microorganism's habitat must be transmitted to the genetic regulatory networks within. Thus, sensing the environment is a well-honed ability in prokaryotes. Figure 3.7 shows a generalized scheme of environmental sensing systems used by prokaryotes. Commonly the sensor is a protein embedded within, but extending out from, the cytoplasmic membrane of the cell. The environmental change causes an allosteric (structural) alteration in protein conformation that leads to its

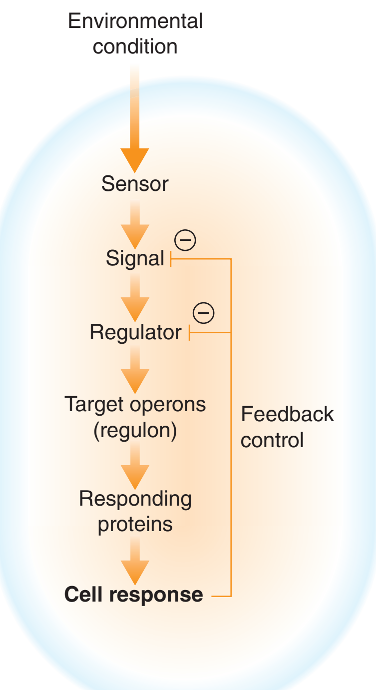


Figure 3.7 Environmental sensing by bacteria. Diagram shows a simplified sensor circuit that transmits signals (such as low nutrient status) to regulatory genes. The eventual protein-mediated cell response completes the negative feedback loop for the circuit. The two right-hand vertical scales set the boundaries for oxidized conditions (top) and reduced conditions (bottom). The left-hand vertical scales juxtaposes a wide variety of oxidative-reduction half reactions: reduced forms (electron donors) are on the right, oxidized forms (electron acceptors) are on the left. See text for how to use this "compass" for predicting electron donor/electron acceptor reactions. (Modified from Niedhardt, F.C., J.L. Ingraham, and M. Schaechter. 1990. *Physiology of the Bacterial Cell: A Molecular Approach*. With permission from Sinauer Associates, Sunderland, MA.)

self-catalyzed binding to a phosphate molecule. The term "sensor kinase" applies. The phosphorylation triggers a subsequent series of phosphorylation events that influence the activity of one or more regulatory proteins. These, in turn, control gene transcription by binding to the promoter or attenuator regions of one or more operons. Following translation of the transcribed genes, protein-catalyzed metabolic changes in the cell eventually deliver negative feedback to the regulatory circuit (Figure 3.7). Overall, the cell's response is matched to the severity of the nutritional stress. Furthermore, when the stress is relieved, the cytoplasmic sensor resumes its previous nonphosphorylated state. Sensor kinase-based control of gene expression is an effective way for cells to respond to environmental cues. The cues may lead to genetic control of large constellations of operons in regulatory networks (regulons). Table 3.5 provides a list of 11 well-characterized

Table 3.5

Prominent gene regulation systems that allow bacteria to sense and coordinate metabolism according to environmental conditions, especially starvation-related stresses. (From Schaechter, M., J.L. Ingram, and F.C. Niedhardt. 2006. *Microbe*. American Society for Microbiology Press, Washington, DC. With permission from the American Society for Microbiology)

Stimulus/ conditions	System	Organism(s)	Regulatory genes (and their products)	Regulated genes (and their products)	Type of regulation
Nutrient utilization					
Carbon limitation	Catabolite repression	Enteric bacteria	*crp* (transcription activator CAP): *cya* (adenylate cyclase)	Genes encoding catabolic enzymes (*lac*, *mal*, *gal*, *ara*, *tna*, *dsd*, *hut*, etc.)	Activation by CAP protein complexed with cAMP as a signal of carbon source limitations
Amino acid or energy limitation	Stringent response	Enteric bacteria and many others	*relA* and *spoT* (enzymes of (p)ppGpp metabolism)	Genes (>200) for ribosomes, other proteins involved in translation and biosynthetic enzymes	(p)ppGpp thought to modify promoter recognition by RNA polymerase
Ammonia limitation	Ntr system (enhances ability to acquire nitrogen from organic sources and from low ammonia concentrations)	Some enteric bacteria	*glnB*, *glnD*, *glnG*, *glnL* (transcriptional regulators and enzyme modifiers)	*glnA* (glutamine synthetase), *hut*, and others encoding deaminases	Complex
Ammonia limitation	Nif system (nitrogen fixation)	*Klebsiella aerogenes* and many others	Multiple genes, including those controlling ammonia assimilation	Multiple genes encoding nitrogenase (for nitrogen fixation)	Complex; ammonia represses activity of NtrC; under low ammonia status, NtrC is active and promotes transcription of NifA, the activator protein for *nif* transcription
Phosphate limitation	Pho system (acquisition of inorganic phosphate)	Enteric bacteria	*phoB* (PhoB, response regulator), *phoR* (PhoR, sensor kinase), *phoU*, and *pstA*, *-B*, *-C*, *-S* (facilitate PhoR function)	*PhoA* (alkaline phosphatase) and ~40 other genes involved in utilizing organophosphates	Two-component regulation; transcriptional activation by PhoB upon signal of low phosphate from the sensor kinase, PhoR

Energy metabolism					
Presence of oxygen	Arc system (aerobic respiration)	*E. coli*	*ArcA* (ArcA, repressor) and *arcB* (ArcB, modulator)	Many genes (>30) for aerobic enzymes	Repression of genes of aerobic enzymes by ArcA upon signal from ArcB of low oxygen
Presence of electron acceptors other than oxygen	Anaerobic respiration	*E. coli*	*fnr* (Fnr)	Genes for nitrate reductase and other enzymes of anaerobic respiration	Transcriptional activation by Fnr
Absence of usable electron acceptors	Fermentation	*E. coli* and other facultative bacteria	Unknown	Genes (>20) for enzymes of fermentation pathways	Unknown
Miscellaneous global systems					
Growth-supporting property of environment	Growth rate control	All bacteria	*fis* (Fis), *hns* (H-NS), *relA* (RelA), and *spoT* (SpoT)	Hundreds of genes, many involved in macromolecule synthesis	Complex; involves availability of RNA polymerase/sigma-70 holoenzyme influenced by passive control
Starvation or inhibition	Stationary phase	All bacteria	*rpoS* (sigma-S), *lrp* (Lrp), *crp* (CAP), *dsrA*, *rprA*, and *oxyS* (regulatory sRNA molecules), and many other regulatory genes	Hundreds of genes affecting structure and metabolism	Multiple modes of regulation in a complex network; involves several global regulatory systems, in addition to selection of genes with promoters recognized by σ^s
Starvation	Sporulation	*Bacillus subtilis* and other spore formers	*SpoOA* (activator), *spoOF* (modulator), and many other regulatory genes	Many (>100) genes for spore formation	Complex; cells respond to nutrient deprivation with SpoOA phosphorelay; a cluster of sigma factors assist in controlling seven stages of differentiation leading to endospore formation and release

cAMP, cyclic adenosine monophosphate; CAP, catabolite activator protein; (p)ppGpp; a mixture of guanosine tetra- and penta-phosphate.

regulatory response systems that allow prokaryotes to contend with nutrient-related environmental conditions.

It is virtually certain that all microorganisms have evolved environmental response systems as sophisticated as those described for model prokaryotes listed in Table 3.5. However, the science of microbiology has not yet progressed far enough to know how widely these specific genetic adaptations have reached through the prokaryotic world. To gain insight into adaptations to starvation found broadly among prokaryotes, we turn to a well-established literature examining the physiological response of microorganisms to starvation. Some of the key scholars addressing the issue of survival of microorganisms in nature, especially under conditions of nutrient limitation and slow growth, include J. Poindexter (1981), H. Jannasch (1969, 1979), D. Roszak and R. Colwell (1987), A. Koch (1971, 1997), D. Button (1998), A. Matin et al. (1989), Y. Henis (1987), S. Pirt (1982), D. Tempest et al. (1983), E. Dawes (1989), R. Kolter et al. (1993), R. Morita (1982, 1997, 2000), Finkle (2006), Hoehler and Jorgensen (2013), and P. Price and T. Sowers (2004). In reviewing the problems of survival of heterotrophic prokaryotes in the marine environment, Morita (1982) proposed five processes that render populations "fit" for starvation survival:

1. All metabolic processes are reduced to a dormant or near-dormant state.
2. When starved, many species will increase in cell number – resulting in reduced cell size (see point 5, below).
3. In the starvation/survival process, any cellular energy reserve material is used to prepare the cell for survival.
4. All metabolic mechanisms are directed to the formation of specific proteins, ATP, and RNA so that the cell, when it encounters a substrate, is equipped to use it immediately without a delay that otherwise would occur if initial amounts of energy had to be expended for the synthesis of RNA and protein. Both RNA and protein synthesis are high-energy-consuming processes, and the high ATP level per viable cell is thus available and used primarily for active transport of substrates across the membrane.
5. The change to a smaller cell size on starvation (miniaturization) permits greater efficiency in scavenging what little energy-yielding substrates there are in the environment and also enhances survival prospects against other adverse environmental factors.

In an essay on bacterial starvation, B. Schink (Lengeler et al., 1999) pointed out that starvation for a carbon (energy) source is very different from starvation for a source of nitrogen, phosphorus, or sulfur because scarcity of mineral nutrients, while preventing an increase in cell mass, may still allow cell maintenance. Should nutrient concentrations diminish substantially, one well-documented cell response is to enhance substrate uptake – either by increasing the number of transport systems (e.g., permeases, ABC-type transporters; see Section 3.2) or shifting to alternate high-affinity systems such as those known for glycerol, phosphate, sugars, and amino acids. Efficient utilization of cellular reserve materials is another

clear survival strategy. The ratio of external availability (supply) versus cellular need (demand) has been emphasized recently by Konopka and Wilkins (2012); these authors have described four key physiological adaptations to nutrient limitation:

(i) Maximize uptake of the rate-limiting element. Molecular mechanisms include derepression of genes encoding the permeases for the limited nutrient and induction of new high-affinity permeases.

(ii) Derepress genes encoding permeases or enzymes that transport or make available alternative forms of the resource. For example, nitrate and amino acids represent alternative N sources to ammonia.

(iii) Downregulate cellular assimilation so that rates do not exceed the inward-flux of the limiting nutrient. This can entail lowering fluxes through pathways that generate monomers for macromolecular synthesis and (most importantly) reducing the capacity for protein synthesis (for example by reducing ribosome content).

(iv) Rearrange metabolism to circumvent bottlenecks caused by the limitations.

Many microorganisms accumulate intracellular storage bodies when nutrients are transiently abundant in the cell's immediate surroundings. Three main classes of cellular reserves are recognized: carbohydrates (polyglucans, glycogen), lipids (poly-β-hydroxy alkanoates, especially poly-β-hydroxybutyrate), and polyphosphates. A fourth type of storage body, cyanophycin, is a nitrogen source (composed of the amino acids arginine and aspartic acid) found in some cyanobacteria. Clearly, when energy, phosphorus, and nitrogen sources are scarce in the external environment, the ability to draw on intracellular reserves offers an advantage in the struggle for survival. The relative importance of uptake versus cellular-reserve survival strategies is undoubtedly dependent upon habitat-specific and organism-specific factors. Nonetheless, Poindexter (1981) perceptively remarked that "over extended periods of time, conservative utilization of nutrients once they are in the cell . . . may be more important than high-affinity uptake systems".

It is important to develop an appreciation for the range of growth rates for microorganisms and the degree to which their survival strategies have been successful. Globally, the significance and ecological success of prokaryotic life in the biosphere are undisputed (see Section 1.2). Detailed insights into this success can be gained by examining habitat-specific trends. Table 3.6 provides estimates of growth rates and survival of microorganisms in nature. The habitats surveyed for growth rate include freshwaters, the ocean, mammals, soil, and the deep subsurface (terrestrial and marine). Despite the fact that the data shown in Table 3.6 reflect approaches and methodologies spanning several decades, several clear patterns emerge. The first is that (compare the first two entries), despite an innate genetic potential to grow with extreme rapidity in laboratory media, *E. coli*'s doubling time in its native habitat (the human intestine) is slowed about 35-fold. This undoubtedly reflects habitat-imposed nutrient limitations. Another contrast in the data shown in Table 3.6 is the immense range of doubling times. Among the 13 nonlaboratory growth-rate entries, the

Table 3.6
Estimates of microbial growth rate, dormancy, and duration of dormancy and survival in nature

Habitat	Organism	Doubling time (DT) or survival time (ST)	References
Growth rate			
Laboratory medium	*E. coli*	20 min DT	Koch, 1971
Human intestine	*E. coli*	12 h DT	Koch, 1971
Mouse	*Salmonella typhimurium*	10–24 h DT	Brock, 1971
Rumen	Heterotrophic bacteria	~12 h DT	Brock, 1971
Pond	Heterotrophic bacteria	2–10 h DT	Brock, 1971
Lake water	Heterotrophic bacteria	10–280 h DT	Brock, 1971
Ocean	Heterotrophic bacteria	20–200 h DT	Jannasch, 1969
Ocean	Autotroph, *Prochlorococcus*	~24 h DT	Vaulot et al., 1995
Soil	Heterotrophs: α Proteobacteria, rhizobia	100 days DT	Gray and Williams, 1971
Shallow groundwater	Heterotrophs: *Acidovorax, Commamonas*	15 days DT	Mailloux and Fuller, 2003
Marine surface sediments	Sulfate reducers	1 year DT	Hoehler and Jorgensen, 2013
Shallow subsurface	*Geobacter*	46 h DT	Holmes et al., 2013
Deep subsurface	Heterotrophs	100 years DT	Phelps et al., 1994; Fredrickson and Onstott, 2001
Deep marine sediments	Sulfate reducers, heterotrophs	200–3000 year DT	Hoehler and Jorgensen, 2013
Duration of dormancy or survival			
Laboratory test tube	*Clostridium aceticum* endospore	34 years ST	Braun et al., 1981
Lake Vostok beneath the Antarctic ice sheet	Dormant nitrifying prokaryotes	$>1.4 \times 10^5$ years ST	Sowers, 2001; Price and Sowers, 2004
Gut of extinct bee trapped in amber	Heterotroph, spore-forming *Bacillus*	$25–40 \times 10^6$ years ST	Cano and Borucki, 1995
Deeply buried clay and shale	Dormant heterotrophs	100×10^6 years ST	Phelps et al., 1994; Price and Sowers, 2004
Precambrian salt crystals	Heterotroph, endospore	250×10^6 years ST	Vreeland et al., 2000

highest (2–10 h) was based on microscopic examination of microcolonies on glass slides immersed in pond water. An extremely slow doubling time of several centuries was estimated for inhabitants of the deep terrestrial subsurface, where low-permeability sediment and nearly constant geochemical conditions place severe limitations on potential growth rates.

The estimate of 200 to 3000 years for turnover time of cells in deep marine sediments (Table 3.6) was reached by modeling rates of sulfate diffusing into sediments and/or rates of viable cells compensating for the chemical decay of amino acids (reviewed by Hoehler and Jorgensen, 2013). In the lower portion of Table 3.6 are three entries attesting to the effectiveness of endospores in conferring longevity to microorganisms. Survival estimates range from a minimum of several decades (in a controlled laboratory setting) to 250×10^6 years, based on the recovery of cells preserved in fluids trapped within an ancient salt crystal. There are also two entries describing dormancy. Sowers (2001) reported that nitrifying prokaryotes residing in liquid veins deep beneath the Antarctic ice sheet had remained viable for $>1.4 \times 10^5$ years. Similarly, deep terrestrial sediments as old as 10^8 years have been found to harbor viable heterotrophic prokaryotes.

Price and Sowers (2004) gained insights into starvation/survival strategies of prokaryotes by reviewing results from more than 30 high-quality studies that emphasized very cold habitats (e.g., ice cores) and deep subsurface sediments (both terrestrial and oceanic). The integrated data set summarized rates of processes carried out by microbial communities in laboratory simulations of nature and from geochemical gradients found in field sites. By plotting rates of microbial metabolism versus temperature, Price and Sowers (2004) were able to discern three distinctive metabolic regimes: growth, maintenance, and survival (dormancy). As expected, energy demand during cellular maintenance is several orders of magnitude lower than for growth, while survival energy demand is orders of magnitude lower than that of maintenance. One shocking finding was that there seems to be no evidence of a minimum temperature for metabolism: even at a temperature of −40 °C in ice, about one turnover of cellular carbon is expected every 10^8 years. Another major conclusion reinforced and extended prior ideas by Morita (2000): extremely slow rates of metabolism characteristic of dormancy work to counteract chemical instability of amino acids (subject to racemization) and nucleic acids (subject to depurination). The extremely low energy requirements for DNA and protein repair during dormancy may be provided to microorganisms in deep, sometimes cold, habitats by slow diffusion of hydrogen gas from the adjacent geologic strata (Morita, 2000; Price and Sowers, 2004).

The evolutionary significance of starvation and chronic energetic stress for microbial life on Earth cannot be overemphasized. Strikingly, this issue appears to be rooted in a major step toward cellular life: encapsulation (Section 2.6). Valentine (2007) has effectively argued that the architectural distinction(s) in the membranes of archael life confer a superior ability to maintain ion gradients. During long-term dormancy, maintenance of ion gradients across membranes (Box 3.1) is crucial for viability and for driving ATP production. The ester-linked bilayer membranes in *Bacteria* are inherently leaky – they are less efficient than the ether-type membranes of *Archaea* in retaining transmembrane gradients of H^+/Na crucial for chemiosmotic energy transduction via ATP synthase (see Section 3.6). Thus,

many membranes of the archael domain are ecologically suited to our planet's ubiquitous nutrient- and energy-deprived habitats.

3.6 A PLANET OF COMPLEX MIXTURES IN CHEMICAL DISEQUILIBRIUM

A theme of this chapter is the generation of ATP, the energy currency of the cell. Without ATP, cell maintenance, cell motility, biosynthetic reactions, replication, cell growth, and heredity would be impossible. The biochemical mechanism of ATP generation relies upon either substrate-level phosphorylation (very significant for many anaerobic microorganisms; Schmitz et al., 2013) or membrane-bound electron transport chains. Electron transport chains create the proton (or sodium) motive force that drives ATP synthases embedded in cytoplasmic membranes (White et al., 2012; Nichols and Ferguson, 2002; Nelson and Cox, 2005; Devlin, 2006; Madigan et al., 2014; Schaechter et al., 2006). ATP-synthase activity relies upon the formation and maintenance of electrochemical potential across intact (non-leaky; see Section 3.5) membranes. However, the ultimate driver of ATP synthesis is the variety of thermodynamically unstable materials that are commingled in the waters, sediments, and soils of the biosphere.

Consider the material present in a liter of seawater, in a handful of soil, or in a scoop of freshwater sediment:

- **What is commingled here? (Answer:** Gases, solids, water, minerals, organic compounds, inorganic compounds, soluble materials, microorganisms, and other life forms – some alive, some perished.)
- **What is the chemical composition of these environmental samples?**
- **What chemical and biochemical reactions are occurring?**
- **Are these mixtures in a state of chemical equilibrium?**

The Earth, its habitats, subhabitats and microenvironments have always been in this complex state consisting of commingled materials. Figure 3.8 provides a global view of our planet. Its status can be summarized as follows: *a heterogeneous mixture of rock, water, gases, and other materials bathed in sunlight*. This is the place where life evolved and where we and microorganisms make a living each day, hour by hour, minute by minute. How do we, as scientists, make sense of biosphere complexity? (For a partial answer, see Section 1.4.) A question more germane to the matter at hand is "How do microorganisms make *physiological* sense of this complexity?" One way to achieve a unified and orderly view of our planet is with thermodynamics. Thermodynamics is the branch of chemistry that rigorously predicts the chemical reactions that are energetically favorable and ones that are not. Thermodynamics can systematically arrange the types of chemical reactions that occur in complex, heterogeneous mixtures typical of biosphere

habitats – we can predict and catalog the energetically favorable reactions. It is these that are the resources which microorganisms (and humans) exploit as energy sources that generate ATP. It is the thermodynamically unstable resources that have provided selective pressure for microbial energy-production strategies throughout evolution. Box 3.3 displays several key thermodynamic principles for predicting chemical and physiological reactions. Box 3.4 elaborates on the principles from Box 3.3 – providing specific examples of half reactions and how the thermodynamics of redox reactions can be used to calculate free energy change.

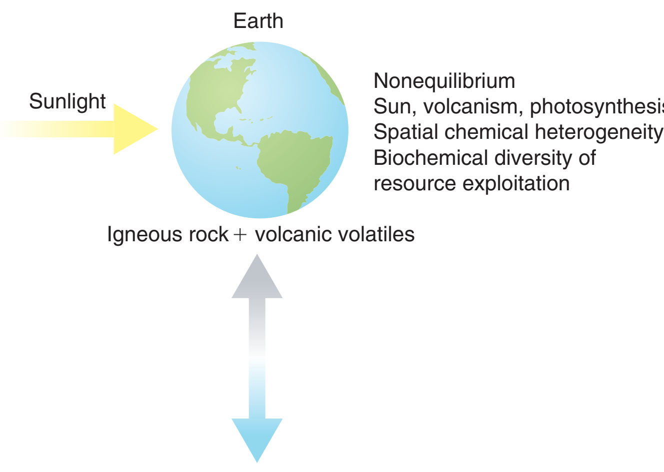


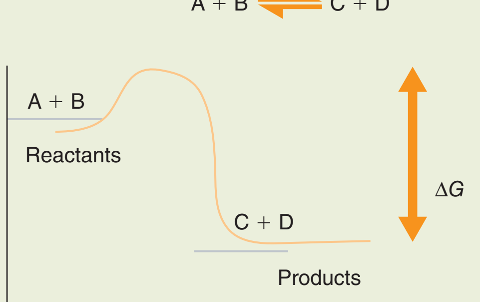
Figure 3.8 Global view of the Earth: a heterogeneous mixture of complex materials (rock, water, gases, others) is maintained in a nonequilibrium state by sunlight and volcanism.

Box 3.3

Thermodynamics: the branch of chemistry that predicts what can happen based on the energy state of reactants and products

In 1877, the American mathematician, Josiah Gibbs, sought to quantify the amount of "useful work" that can be harnessed during completion of chemical (or other) reactions.

$$A + B \rightleftharpoons C + D$$



Types of reactions

Changes in state
Dissolution/precipitation
Complexation
Acid/base
Sorption/desorption
Oxidation/reduction (key for biological systems to generate ATP)

Products − reactants = $-\Delta G$ (free energy)

$$\Delta G = \Delta G^0 + RT \frac{[C]\,[D]}{[A]\,[B]}$$

ΔG = free-energy change under conditions specified
$\Delta G°$ = free-energy change under standard conditions of 1 atm pressure and 1 m concentration
T = temperature (degrees K)
R = universal gas constant (8.29 J/mol/K)

Box 3.4

Four stages to understanding oxidation/reduction reactions, chemical equilibrium, and free energy

Here we use rust and metabolism to define electron flow and to calculate free energy yields.

Stage 1. Defining oxidation–reduction reactions

Rust formation (iron metal) $4Fe^0 + 3O_2 \rightarrow 2Fe_2^{3+}O_3^{2-}$ (rust)

Electron flow: $4Fe^0 \rightarrow 4Fe^{3+} + 12e^-$ (electron donor)

$12e^- + 3O_2^0 \rightarrow 6O^{2-}$ (electron acceptor)

Combustion and respiration $CH_2O + O_2 \rightarrow CO_2 + H_2O$

Electron flow: $C^0 \rightarrow C^{4+} + 4e^-$ (electron donor)

$4e^- + O_2 \rightarrow 2O^{2-}$ (electron acceptor)

Stage 2. An example of balancing a redox equation

What is the balanced reaction for the oxidation of H_2S to SO_4^{2-} by O_2 (modified from Madigan et al., 2014)?

1 *Electron donor half reaction*. First, decide how many electrons are involved in the oxidation of H_2S to SO_4^{2-}. This can be easily calculated using simple arithmetic from rules of charge balance and the fixed and/or limited oxidation states of the common atoms. Because H has an oxidation state of +1, the oxidation state of S in H_2S is −2. Because the reduced form of O has an oxidation state of −2, the oxidation state of S in SO_4^{2-} is +6. Thus, the oxidation of S^{2-} to SO_4^{2-} involves an eight-electron transfer (S changes from −2 to +6):

$$S^{2-} \rightarrow SO_4^{2-} + 8e^-$$

2 *Electron-acceptor half reaction*. Because each O atom in O_2 can accept two electrons (the oxidation state of O in O_2 is zero, but in H_2O is −2), this means that two molecules of molecular oxygen, O_2, are required to provide sufficient electron-accepting capacity to accommodate the eight electrons from S^{2-}:

$$8e^- + 2O_2 \rightarrow 4O^{2-}$$

Thus, at this point, we know that the reaction requires 1 H_2S and 2 O_2 on the left side of the equation and 1 SO_4^{2-} on the right side. To achieve an ionic balance, we must have two positive charges on the right side of the equation to balance the two negative charges of S^{2-} and SO_4^{2-} Thus $2H^+$ must be added to the both sides of the equation, making the overall reaction:

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$$

By inspection, it can be seen that this equation is balanced in terms of the total number of atoms of each kind on each side of the equation.

In general for microbiological reactions, the first step is quantitatively balancing electron flux between donor and acceptor. Next, mass balances of O can be achieved by adding H_2O to the side of the reaction with an O deficit. Then, the third and final step involves adding H^+ to the other side to compensate for an H deficit. *Because all reactions take place in an aqueous medium, H^+ production shows that a reaction generates acidity while H^+ consumption indicates generation of alkalinity.*

Stage 3. Examples of calculating free energy yields

Chemical reactions under standard conditions (for chemists)

The procedures to calculate ΔG for chemical reactions are well established (e.g., Dolfing, 2003; Madigan et al., 2014). Briefly, calculations of the changes in Gibbs free energy of a system are made according to the equation:

$$\Delta G^\circ = \Sigma G_f^o \text{ (products)} - \Sigma G_f^o \text{ (reactants)}$$

The naut sign (°) indicates that the calculations are made for standard conditions, i.e., concentrations of 1 M, temperature of 25°C, and a partial pressure of 1 atm for gases. This approach is appropriate for the reaction between H_2S and O_2 shown above.

Once an equation has been balanced, the free energy yield can be calculated by inserting the values for the free energy of formation of each reactant and product from standard tables (Stumm and Morgan, 1996; Dolfing, 2003; Madigan et al., 2014). For instance, for the equation:

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$$

$$\rightarrow G_f \text{ values} \rightarrow (-27.87) + (0) \rightarrow (-744.6) + 2(-39.83) \text{ (assuming pH 7)}$$

$$\Delta G_f^o = -796.39 \text{ kJ/reaction}$$

The G_f values for the products (right side of the equation) are summed and the G_f values for the reactants (left side of the equation) are subtracted, taking care to ensure that the arithmetic signs are correct.

Biochemical reactions under physiological conditions (for microbiologists)

For evaluations of changes of free energy under actual physiological conditions, many assumptions made by chemists are not relevant. Under environmentally relevant conditions, the concentrations of substrates and products are not 1 M and the partial pressures are not 1 atm. One way to compensate for deviations from standard conditions is to adjust calculations according to the Nernst equation, because the change in Gibbs free energy values of a reaction are directly linked to its equilibrium constant. This is reflected in ΔG values. For a hypothetical reaction:

$$aA + bB \rightarrow cC + dD$$

ΔG values are calculated by using the mass equation:

$$\Delta G = \Delta G^o + RT \ln[C]^c[D]^d/[A]^a[B]^b$$

where R is a constant (8.29 J/mol/K) and T is temperature in K.

Under dynamic biological conditions that feature very low concentrations of some reactants and products, free energy yields can shift drastically from those predicted under standard conditions.

Box 3.4 *Continued*

Stage 4. A preferred approach to calculating biochemical free energy yield using electron potential (see also Figure 3.10 later in Section 3.8)

Reduction potentials of many redox half reaction pairs are shown graphically in Figure 3.10. The amount of energy that can be released from two half reactions can be calculated from the differences in reduction potentials of the two reactions and from the number of electrons transferred. The further apart the two half reactions are and the greater the number of electrons, the more energy is released. The conversion of potential difference to free energy can be calculated using another version of the Nernst equation with the formula $\Delta G^{\circ\prime} = -n\mathrm{F}\Delta E_o'$, where n is the number of electrons, F is the Faraday constant (96.48 kJ/V), ΔE_o is the difference in potentials (electron acceptor minus electron donor), and the prime (′) symbol normalizes all reactions to a common pH of 7.0. Energetically favorable oxidations are those in which the flow of electrons is from (reduced) electron donors (lower right in Figure 3.10 in Section 3.8) up to (oxidized) electron acceptors (upper left in Figure 3.10).

Example: hydrogen gas oxidation by microorganisms using molecular oxygen and nitrate as terminal electron acceptors

The couple $2H^+/H_2$ has the potential of −0.41 V and the $^1/_2O_2/H_2O$ pair has a potential of +0.82 V. The potential difference is 1.23 V, which (because two electrons are involved) is *equivalent to a free energy yield* (ΔG°) *of −237.34 kJ* [= (−2)(96.48 kJ/V)(1.23 V)].

By contrast, if oxygen is unavailable and anaerobic nitrate respirers (producing nitrite) are active, the potential difference between the $2H^+/H_2$ and the NO_3^-/NO_2^- reactions is 0.84 V, which is *equivalent to a free energy yield of −162.08 kJ* [= (−2)(96.48 kJ/V)(0.84 V)].

Because many biochemical reactions are two-electron transfers, it is often useful to give energy yields for two-electron reactions, even if more electrons are involved. Thus, the SO_4^{2-}/H_2 redox pair involves eight electrons and complete reduction of SO_4^{2-} with H_2 requires $4H_2$ (equivalent to eight electrons). From the reduction potential difference between $2H^+/H_2$ and SO_4^{2-}/H_2S (0.19 V), a free energy yield of −146.64 kJ is calculated, or −36.66 kV per two electrons:

$$4H_2 + SO_4^{2-} \rightarrow 4H_2O \text{ (as written, eight electrons are transferred)}$$
$$\Delta G^{o\prime} = -n\mathrm{F}\Delta \mathrm{E}_o{}' : \quad \Delta G^{o\prime} = (-8e)\quad (96.48\,\mathrm{kJ/V})(0.19\ \mathrm{V}) = -146.64\,\mathrm{kJ}$$
$$\text{Normalized to } 2e^- : \quad \Delta G^{o\prime} = (-2e)\quad (96.48\,\mathrm{kJ/V})(0.19\ \mathrm{V}) = -36.66\,\mathrm{kJ}$$

One of the major features of the information conveyed in Figure 3.8 is that Earth is in a dynamic state of constant disequilibrium. Key inputs of energy to the biosphere are light from the Sun and a slow release of heat and materials from the Earth's core via volcanism. These global energy inputs are imposed upon a spatially varied tapestry of elevation (from the deepest ocean trench to the summit of Mt. Everest), latitude (from equator to the poles), climate, and chemical conditions (pH, salinity, oxygen concentration, other gases, etc.) spanning both aquatic and terrestrial habitats around the globe. Details of habitat diversity are presented in Chapter 4.

3.7 A THERMODYNAMIC HIERARCHY DESCRIBING BIOSPHERE SELECTIVE PRESSURES, ENERGY SOURCES, AND BIOGEOCHEMICAL REACTIONS

In Chapter 2, we emphasized that the development of photosynthesis and the photosynthetic apparatus was a major event in evolution – it allowed life to harvest light energy from the Sun, led to the accumulation of fixed carbon as biomass, and created a global pool of gaseous oxygen (that itself had vast metabolic and evolutionary repercussions). Thermodynamics helps to systematically define the far-ranging influence of photosynthesis upon biogeochemical processes. Figure 3.9, devised by Zehnder and Stumm (1988), presents an insightful portrait of the Earth's biogeochemistry. In a single figure these biogeochemists were able to create a conceptual masterpiece that captures the essence of mechanistic relationships between oxidation–reduction processes and ecosystem function. On the left-hand side of Figure 3.9 is an arrow representing sunlight impinging on the Earth. As sunlight drives photosynthesis, water is split into oxygen gas (arrow up to the O_2

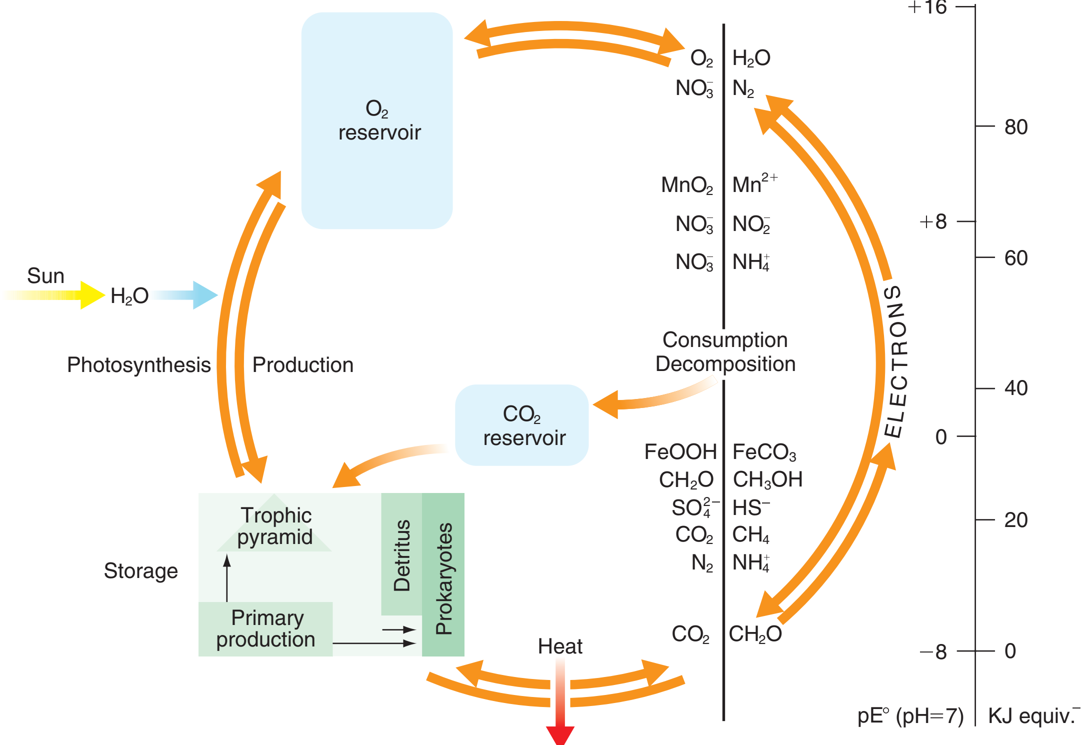


Figure 3.9 A global thermodynamic model of the biosphere. Sunlight and ecosystem processes are arranged along a vertical scale of oxidation–reduction potential. (From Zehnder, A.J.B. and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. In: A.J.B. Zehnder (ed.), *Biology of Anaerobic Microorganisms*, pp. 1–38. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York.)

reservoir) and reducing power (arrow down leading to reduced carbon in the box labeled storage, which surrounds primary production, trophic pyramid, detritus, and prokaryotes). By capturing sunlight and simultaneously creating pools of both oxygen and reduced carbon in biomass, photosynthesis assures that our planet is in a state of disequilibrium. Photosynthesis effectively (very effectively) reverses the universal drift toward thermodynamic equilibrium on our planet (Zehnder and Stumm, 1988; Stumm and Morgan, 1996). *It is the commingling of reduced biomass with oxygen and other oxidized compounds* (such as nitrate, Fe oxides, Mn oxides, sulfate, and CO_2) *that sets the stage for metabolism by chemosynthetic organotrophic life* (see Section 3.3). Likewise the commingling of reduced inorganic compounds (e.g., hydrogen gas, NH_3, H_2S) with oxygen and other oxidized materials sets the stage for metabolism by chemosynthetic lithotrophic life. A nonequilibrium state is created by co-occurring pairs of reduced and oxidized materials in waters, sediments, and soils of the biosphere. These very same pairs of reduced and oxidized materials constitute the selective pressures that have driven evolution of ATP generation in nonphotosynthetic organisms since life began. To the right of Figure 3.9 is a vertical scale of oxidation/reduction half reactions arranged with O_2/H_2O at the top and CO_2/CH_2O at the bottom. An expanded view of this vertical scale (hierarchy) is discussed further below. The figure also features a vertical scale whose units are pE and kilojoules of energy per electron transferred in a reaction. The pE values range from a low of −8 to a high of +16. As described in Box 3.5, pE is to electrons what pH is

Box 3.5

Understanding pE: an analogy to pH that is the vertical scale of the oxidation/reduction hierarchy (Figures 3.9 and 3.10) and describes the oxidation/reduction status of biosphere habitats

$pH = -\log[H^+]$	$pE = -\log\ [e^-]$
$HA = H + A^-$	$Red = Ox + e^-$
$K = \frac{[H^*][A^-]}{[HA]}$	$K = \frac{[Ox][e^-]}{[Red]}$
$pH = pK + \log\frac{[A]}{[HA]}$	$pE = pK + \log\frac{[Ox]}{[Red]}$
pH = measure of a solution's tendency to donate or accept protons	pE = measure of a solution's tendency to donate or accept electrons

HA = protonated form of the conjugate base, A^-; A^- = conjugate base (deprotonated); Red = reduced form of compound; Ox = oxidized form of compound; e^- = electron

to protons; pE is an index of how oxidized or how reduced a given setting may be. Just as a high pH value (e.g., 12) indicates an exceedingly low concentration of protons in solution, a high pE indicates rareness of electrons. Rareness of electrons is synonymous with oxidizing conditions and the converse is synonymous with reducing conditions.

In any habitat with a significant partial pressure of oxygen gas, the pE is poised at a value of about +14. For this reason, both the oxygen reservoir and the O_2/H_2O half reaction are placed at the top of Figure 3.9. If oxygen is absent from a system (e.g., the prebiotic Earth or contemporary anaerobic freshwater sediments) the pE drops to the next highest redox half reaction that happens to predominate in the habitat of interest. Depending on local geochemical conditions and ecological processes, the dominant oxidation–reduction half reaction may be NO_3^-/N_2, $FeOOH/FeCO_3$ or CO_2/CH_2O. Note that in Figure 3.9, the "storage box" of reduced organic carbon and the CO_2/CH_2O redox couple occur at the same height on the pE redox scale. From a thermodynamic viewpoint, the life forms that dwell in the biosphere are simply reservoirs of reduced organic carbon, waiting to be oxidized.

3.8 USING THE THERMODYNAMIC HIERARCHY OF HALF REACTIONS TO PREDICT BIOGEOCHEMICAL REACTIONS IN TIME AND SPACE

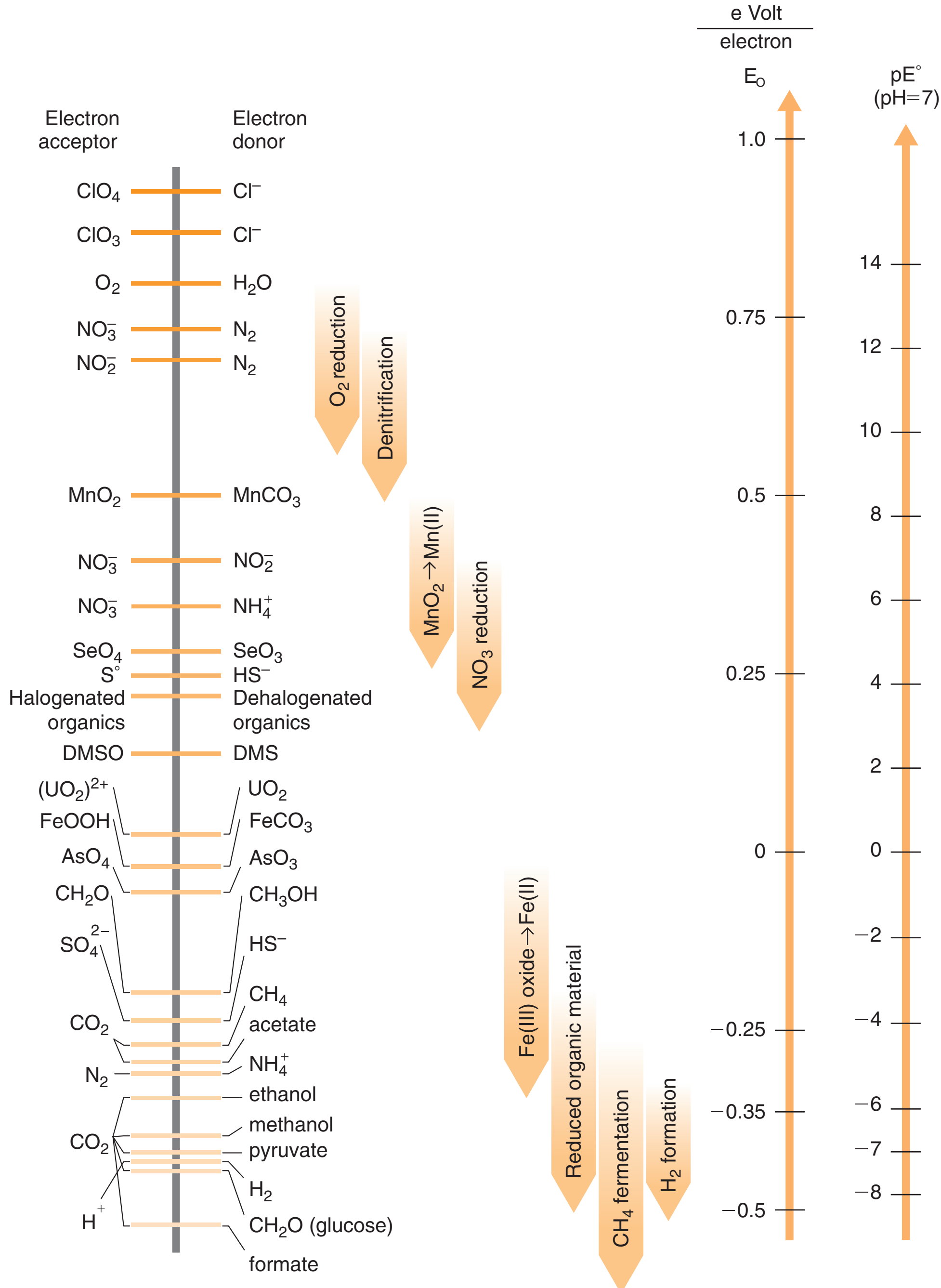
Figure 3.10 shows an expanded view of the scale of half reactions in Figure 3.9. This diagram can be considered a general guide for predicting and interpreting a large portion of the microbially mediated ATP-generating biochemical reactions in the biosphere. The information in Figure 3.10 is a tool that can help us maneuver through the physiological maze of reactions – in this sense, the figure is a "compass for environmental microbiologists".

Figure 3.10 graphically depicts the relationship between reduced and oxidized substrates as a vertically arranged hierarchy of oxidation–reduction half reactions. The vertical axes are electron potential (as E_0) and pE. Compounds on the left of the half reaction hierarchy are in an oxidized state (electron acceptors), while those on the right are in the reduced form (electron donors). Furthermore, the transition from oxidized to reduced forms is governed by the redox status of the system of interest and by catalytic mechanisms of microbially produced enzyme systems. Highly oxidizing conditions appear in the upper portion of the hierarchy in Figure 3.10, while highly reducing conditions are listed in the lower portion. This figure can be used to predict which combinations of half reaction pairs are thermodynamically possible because, under standard conditions, the lower reaction proceeds leftward (electron producing) and the upper reaction proceeds rightward (electron accepting). Focus on the half-reaction

hierarchy portion of Figure 3.10. Graphically, pairs of thermodynamically favorable half reactions can be linked simply by drawing arrows diagonally from the lower right to the upper left portion of the hierarchy. Fundamental reactions of the carbon cycle tie the oxidation of photosynthetically produced organic carbon (e.g., CH_2O; lower right of the half reaction hierarchy in Figure 3.10) to the variety of final electron acceptors that may be present in natural habitats (O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2). Each of these coupled half reactions is mediated by chemosynthetic organotrophic microorganisms. Moreover, when diagonal arrows directing carbohydrate oxidation to the reduction of these electron acceptors are drawn, the length of each arrow is proportional to the free energy gained by the microorganisms. Thus, microorganisms metabolizing carbohydrates with O_2 as a final acceptor (CH_2O is oxidized to CO_2, while O_2 is reduced to H_2O) are able to generate more ATP than those carrying out nitrate respiration. These microorganisms, in turn, gain more energy than those using Mn^{4+} and Fe^{3+} as final electron acceptors. This pattern continues down the hierarchy of electron-accepting regimes until methanogenesis (CO_2 as the final electron acceptor) is reached. There is a three-way convergence between the thermodynamics of half reactions, the physiology of microorganisms, and the presence of geochemical constituents actually found in field sites. It is notable that synthetic halogenated compounds (such as tetrachloroethene and polychlorinated biphenyls) are also present in the hierarchy depicted in Figure 3.10. Halogenated compounds can be utilized as final electron acceptors by microorganisms (see also Section 8.3). Other oxidation–reduction half reactions of important inorganic and other environmentally important compounds (e.g., arsenate, selenite, ferrous iron, uranium (VI) oxycation, dimethyl sulfoxide (DMSO), and perchlorate) also appear in Figure 3.10.

The beauty of the scheme presented in Figure 3.10 is that it makes sense of what might be perceived as the overwhelming complexity of real-world conditions that prevail in aquatic and terrestrial environments. The predictions work for two reasons: (i) the power of thermodynamics and (ii) the fact that only a few forms of a few elements (e.g., C, O, N, H_2, Fe, Mn) are prevalent

Figure 3.10 The hierarchy of half reactions, between electron donors and electron acceptors, defines biogeochemical reactions carried out by microorganisms in waters, soils, and sediments. The two right-hand vertical scales set the boundaries for oxidized conditions (top) and reduced conditions (bottom). The far-left vertical axis places a wide variety of oxidation–reduction half reactions on the redox scale. For each half reaction, reduced forms (electron donors) are on the right and oxidized forms (electron acceptors) are on the left. See text for how to use this "compass" for predicting electron donor/electron acceptor reactions. Abbreviations: DMS, dimethylsulfide; DMSO, dimethylsulfoxide. (Modified from Zehnder, A.J.B. and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. In: A.J.B. Zehnder (ed.), *Biology of Anaerobic Microorganisms*, pp. 1–38. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York.)



Electron acceptor
Electron donor
ClO_4 Cl^-
ClO_3 Cl^-
O_2 H_2O
NO_3^- N_2
NO_2^- N_2
MnO_2 $MnCO_3$
NO_3^- NO_2^-
NO_3^- NH_4^+
SeO_4 SeO_3
S° HS^-
Halogenated organics
Dehalogenated organics
DMSO DMS
$(UO_2)^{2+}$ UO_2
FeOOH $FeCO_3$
AsO_4 AsO_3
CH_2O CH_3OH
SO_4^{2-} HS^-
CO_2
CH_4
acetate
N_2
NH_4^+
ethanol
CO_2
methanol
pyruvate
H^+
H_2
CH_2O (glucose)
formate
O_2 reduction
Denitrification
$MnO_2 \rightarrow Mn(II)$
NO_3 reduction
Fe(III) oxide→Fe(II)
Reduced organic material
CH_4 fermentation
H_2 formation
e Volt / electron
E_O
1.0
0.75
0.5
0.25
0
−0.25
−0.35
−0.5
pE° (pH=7)
14
12
10
8
6
4
2
0
−2
−4
−6
−7
−8

Science and the citizen

Microorganisms can breathe using chlorinated solvents

Headline news: groundwater pollution by chlorinated solvents, PCE, and TCE, and reductive dechlorination by microorganisms

$Cl_2C{=}CCl_2$ is the chemical formula for tetrachloroethene (also known as perchloroethene, perchloroethylene, and PCE). $HClC{=}CCl_2$ is the chemical formula for trichlorothene (also known as trichloroethylene and TCE). For many decades, these synthetic compounds have been used widely in the dry cleaning, metal machining, and electronics industries. PCE and TCE are effective at leaving surfaces very clean – a critical need in many manufacturing processes. But both PCE and TCE, suspected carcinogens, have been improperly handled. These compounds are among the most ubiquitous groundwater pollutants in the world. They have a density greater than 1 g/cm^3. This means that, after being spilled, they penetrate the soil and sediment and reach groundwater. They then continue to sink and become nearly intractable in geologic formations below. Once released to a subsurface habitat, pools of TCE and PCE can seldom be retrieved and their slow dissolution can contaminate huge volumes of adjacent flowing groundwater.

SCIENCE: Microorganisms to the rescue?

In standard *aerobic* biodegradation tests, PCE and TCE are generally found to be nonbiodegradable. The carbon atoms in PCE and TCE are not good electron donors for aerobic microorganisms. However, under *anaerobic* conditions it has been discovered that PCE and TCE are microbiologically useful, physiological electron acceptors (McCarty, 1997). This discovery was made both in the laboratory and in field sites. In laboratory-incubated bottles, anaerobic microorganisms from groundwater sediment and sewage sludge were found to consume PCE and TCE when the microorganisms were supplied with electron donors like methanol and hydrogen gas. As PCE was consumed, TCE appeared. Then, sequentially, new compounds appeared: dichloroethene (DCE), monochloroethene (vinyl chloride or VC) and ethene. This process has become known as sequential "reductive dechlorination" or "halorespiration". As hydrogen atoms and electrons are added to the two-carbon ethene backbone of the pollutant molecules (as reduction occurs), the molecules are gradually stripped of their chlorine atoms.

In field sites where PCE and TCE were spilled along with electron donors (like methanol), the same series of metabolites can be found. Initially, spilled chemicals and groundwaters are free of DCE, VC, and ethene. Yet as time passes, DCE, VC, and ethene appear as groundwater constituents.

This is biogeochemistry in action. Microbially mediated, reductive dechlorination has the potential to counteract groundwater pollution by chlorinated solvents.

- **Is microbially mediated reductive dechlorination of PCE and TCE always reliable and 100% beneficial?**

Answer: Unfortunately, the answer is "not always".

The figure below (from McCarty, 1997) provides an overview of many of the physiological and ecological factors that govern if and when reductive dechlorination will be complete. As shown in Figure 3.12 in Section 3.10, anaerobic processes are often carried out by many cooperating populations that constitute cooperative food chains. Organic materials such as those derived from plant biomass (see Section 7.3) are fermented in anaerobic habitats, leading to transient extracellular pools of hydrogen and acetate. The hydrogen and acetate are used directly as electron donors by at least four key functional groups of microorganisms: sulfate reducers, iron reducers, methanogens, and reductive dechlorinators. Understanding how the four physiological groups of anaerobic populations compete for electron donors is an important goal in managing contaminated sites in ways that optimize reductive dechlorination.

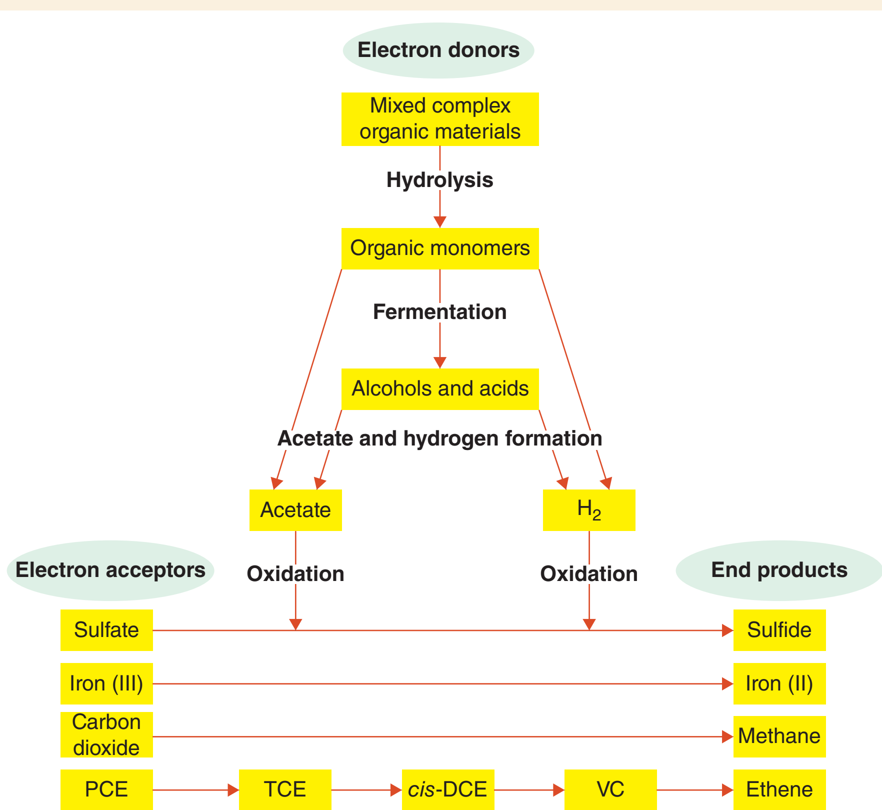


Figure 1 Detoxification: a competitive situation, showing the electron flow from electron donors to electron acceptors in the anaerobic oxidation of mixed and complex organic materials. Microorganisms that can use chlorinated compounds (PCE, TCE, *cis*-DCE, and VC) as electron acceptors in reductive dechlorination compete for the electrons in the acetate and hydrogen intermediates with microorganisms that can use sulfate, iron (III), and carbon dioxide. (From McCarty, P.L. 1997, Microbiology: breathing with chlorinated solvents. *Science* **276**:1521–1522. Reprinted with permission from AAAS.)

Note that along the bottom of the figure, the endproduct is ethene – a nontoxic, naturally occurring compound. However, the immediate precursor of ethene is VC, a proven carcinogen. Thus, if the reductive dechlorination process stops short, it may convert suspected carcinogenic compounds (PCE and TCE) to a known carcinogen (VC). Clearly, then, there are some risks in applying reductive dechlorination-based strategies in technologies aimed at environmental cleanup of contaminated sites (for more information on biodegradation and bioremediation, see Section 8.3). Ongoing research continues to address scientific issues pertinent to the biogeochemistry, the molecular microbial ecology, and the genomics of microorganisms capable of metabolizing PCE, TCE, and their daughter products (e.g., Maymo-Gatell et al., 1997; He et al., 2003; Seshadri et al., 2005; Löffler et al., 2013).

Research essay assignment

PCE and TCE were used industrially for many years before the environmental and health hazards they pose were recognized. Prepare an essay that documents the sequence of events linking improper disposal of chlorinated solvents to legislation recognizing their environmental threats. Next, tie in the discovery of microbial cleanup technologies. Base the essay on a search of the scientific and news literature.

in biosphere habitats. Table 3.7 formally defines the eight common processes that are recognized to occur in carbon-rich habitats. These coupled biogeochemical reactions are: aerobic respiration, denitrification, Mn reduction, Fe reduction, fermentation, sulfate reduction, methanogenesis, and acetogenesis. The hierarchy of the reactions is clear: aerobic respiration occurs at a pE of 13.75 and its free energy yield exceeds that of the other reactions. Thus, in a given aquatic or terrestrial habitat rich in carbon, microorganisms endowed with the physiological capacity to carry out aerobic respiration will have an advantage – their ATP-generating ability exceeds that of the other microbial residents. As long as the supply of oxygen is adequate, aerobic respiration will predominate. Once the supply of oxygen is exhausted, nitrate-respiring organisms will become predominant … and so on down the hierarchy. This predictable sequence of physiological processes is reinforced at the level of gene regulation within individual microbial cells. Generally speaking, the final electron acceptor allowing the highest free energy yield inhibits expression of genes required for utilization of final electron acceptors residing lower in the hierarchy (Saffarini et al., 2003; Gralnick et al., 2005). The reader should be aware that kinetic considerations, such as substrate uptake affinities by microorganisms and biochemical reaction rates, can also be important in determining which electron-accepting process will predominate in a given habitat. Vital for anaerobic physiology and food chains (see Section 3.10), hydrogen gas production occurs via reduction of protons. This process (mediated by hydrogenase enzymes) may be energetically unfavorable, but allows cells to dispose of excess reducing power, thereby maintaining redox balance (Hedderich and Forzi, 2005).

Table 3.7

Hierarchy of oxidation–reduction processes typical of carbon-rich environments. When carbonaceous materials (CH_2O) are electron donors, individual microorganisms or consortia of populations can mediate electron transfer reactions. See also Figures 3.10 and 3.12. (Modified from Stumm, W. and J.J. Morgan. 1996. *Aquatic Chemistry: Chemical equilibria and rates in natural waters*, 3rd edn. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York)

		PE regimes		
Process		**($PE^\circ \approx \log K$)**	**Heterotrophic reactions**	**ΔG° (kJ/eq.)**
Aerobic respiration	$^1/_4O_2\ (g) + H^+ + e = {}^1/_2H_2O$	+13.75	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	−125
Denitrification	$^1/_5\ NO_3^- + {}^6/_5H^+ + e = {}^1/_{10}N_2 + {}^3/_5H_2O$	+12.65	$5CH_2O + 4NO_3^- + 4H^+ \rightarrow 5CO_2 + 2N_2 + 7H_2O$	−119
Manganese reduction	$^1/_2MnO_2(s) + {}^1/_2\ HCO_3^- + {}^3/_2H^+ + e = {}^1/_2MnCO_3(s) + H_2O$	+8.9	$CH_2O + 2MnO_2 + 4H^+ \rightarrow CO_2 + 2Mn^{2+} + 3H_2O$	−98
Iron reduction	$FeOOH(S) + HCO_3^- + 2H^+ + e = FeCO_3(s) + 2H_2O$	−0.8	$CH_2O + 4FeOOH + 8H^+ \rightarrow CO_2 + 4Fe^{2+} + 7H_2O$	−42
Fermentation	$^1/_2CH_2O + H^+ + e = {}^1/_2CH_3OH$	−3.01	$3CH_2O \rightarrow CO_2 + CH_3CH_2OH$	−27
Sulfate reduction	$^1/_8\ SO_4^{2-} + {}^9/_8H^+ + e = {}^1/_8H_2S(g) + {}^1/_2H_2O$	−3.75	$2CH_2O + SO_4 + 2H^+ \rightarrow 2CO_2 + H_2S + 2H_2O$	−25
Methanogenesis	$^1/_8CO_2(g) + H^+ + e = {}^1/_8CH_4(g) + {}^1/_4H_2O$	−4.13	$2CH_2O \rightarrow CO_2 + CH_4$	−23
Acetogenesis	$^1/_4CO_2(g) + H^+ + e = {}^1/_8CH_3COOH + {}^1/_4H_2O$	−4.2	$2CH_2O \rightarrow CH_3COOH$	−22

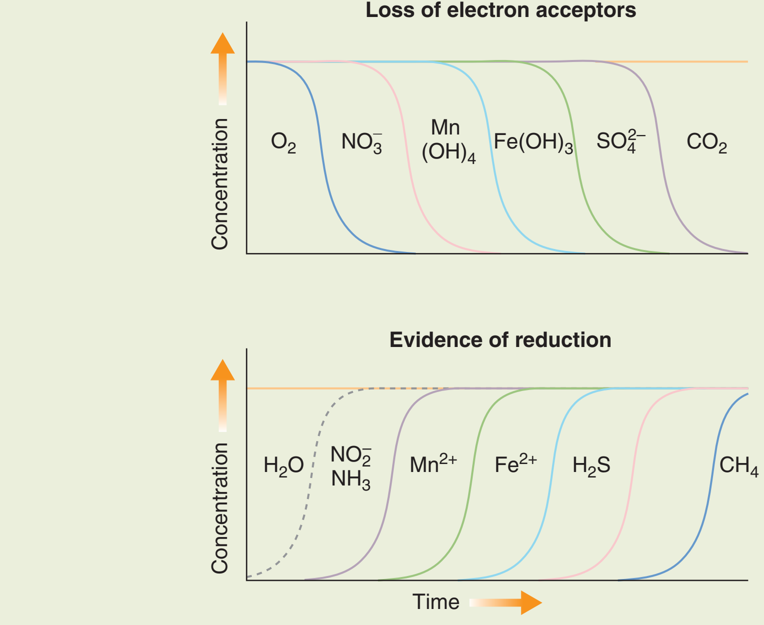
The information in Table 3.7 accurately represents the energetics of a selection of major (not all) biogeochemical processes driven by carbon as an electron donor; other processes such as dissimilatory reduction of nitrate to ammonia (DNRA) and anaerobic ammonium oxidation (anammox) are presented in Sections 7.3 to 7.5. Note that the information in Table 3.7 overlooks both the biochemical and ecological details of the hierarchy; examples of such details, especially the involvement of anaerobic food chains, are presented in Sections 3.10 and 7.3 to 7.5. The power of the information embodied by the thermodynamic "compass" (Figure 3.10) can be illustrated by its ability to often successfully predict, in time and space, what biogeochemical reactions will occur. A laboratory experiment, illustrating the sequence of physiological processes (also known as terminal electron acceptor processes, TEAPs) is described in Box 3.6. The rationale of the experiment is to provide the heterotrophic soil community of microorganisms with an excess supply of electron donor (potato starch) but limited supplies of common electron acceptors (O_2, NO_3^-, $Mn(OH)_4$, $Fe(OH)_3$, SO_4^{2-}, CO_2). If water samples are removed and analyzed over time, the thermodynamically predicted patterns shown in Box 3.6 emerge. The most energetically favorable reactions occur first and the least favorable last. The sequence of depleted electron acceptors is O_2, NO_3^-, Mn^{4+}, Fe^{3+}, and SO_4^{2-}. Each electron acceptor is converted to its reduced form. In an aqueous medium, newly formed H_2O would not be detectable (unless the original O_2 were labeled with the stable isotope ^{18}O and analyses were performed with a mass spectrometer). However, if the TEAP experiment is carefully implemented, reduced forms of the other acceptors can be measured. Nitrate is converted by denitrifying microorganisms first to nitrite and then to nitrogen gas (an alternative endproduct for some microorganisms is ammonia; see Sections 7.3 and 7.4). $Mn(OH)_4$, a solid mineral, is converted to soluble Mn^{2+} by manganese reducers. $Fe(OH)_3$, also a solid mineral, is converted to soluble Fe^{2+} by iron reducers. Sulfate-reducing bacteria convert sulfate anions to H_2S, a material that smells like rotten eggs and that readily reacts with many metal ions to form a black precipitate. Finally, methanogenic bacteria convert CO_2 to methane. Carbon dioxide is universally present in heterotrophic microbial systems as a result of its formation during respiration (though there may be exceptions: for example, in high pH environments, CO_2 availability may be drastically reduced). This endogenous source of CO_2 accounts for the constancy of CO_2 in the top panel of Box 3.6, and also assures the potential utilization of this often energetically least favorable major electron acceptor in all anaerobic habitats.

Acetogenesis is a process that coexists with methanogenesis in many anaerobic environments. Many acetogenic bacteria are chemolithoautotrophs that carry out reactions between the following electron donor/acceptor pairs: H_2/CO_2, CO/CO_2, and H_2/CO. However, acetogens are metabolically versatile – able to utilize a variety of electron donors and acceptors and to utilize fixed carbon sources when available (Drake et al., 2006). A key trait

Box 3.6

Laboratory demonstration of the sequence of thermodynamically predicted physiological processes that generate proton-motive force, and hence ATP

- *Experimental design*: closed vessels containing water (1 L), 1 g potato starch, dissolved O_2, NO_3^-, SO_4^{2-}, $Fe(OH)_3$, $Mn(OH)_4$, CO_2, and soil (10 g).
- *Rationale*: provide carbon and energy for heterotrophs. Potato starch is the electron donor. Oxygen and other electron acceptors are provided.
- *Measure*: time course of geochemical change.



of acetogens is production of acetic acid via the Wood–Ljungdahl pathway that relies upon the enzyme acetyl-CoA synthase for CO_2 fixation and in terminal electron-accepting energy conservation (Drake et al., 2006). Factors that regulate and control the relative importance of methanogenesis and acetogenesis in anaerobic habitats are not fully understood. Based on simple energetic consideration, methanogenesis from H_2 is a more favorable process than acetogenesis (−136 kJ/mol versus −105 kJ/mol, respectively; Table 3.7). However, in many environments (e.g., termite gut, microaerophilic zones in soil or sediment), acetogens can compete successfully with methanogens by positioning themselves closer to the H_2 source or by supplementing their nutrition with fixed organic compounds or by tolerating exposure to oxygen. At low temperatures and low pH (e.g., habitats such as tundra wetland soils)

acetogenic populations can flourish. Furthermore, in high-temperature habitats featuring high acetate concentrations (e.g., anaerobic digesters), acetogens and methanogens can form a syntrophic relationship that converts acetate to methane (Schink, 1997; Schink and Stams, 2013). Regardless of which is the dominant process, the metabolic activities of both methanogens and acetogens are clearly a very important part of the global carbon cycling and anaerobic food chains in virtually all ecosystems.

The laboratory demonstration of redox reaction chronology (Box 3.6) has been manifest innumerable times in real-world contaminated field sites. Box 3.7 provides an example of a groundwater contamination scenario that leads to spatially distinctive zones of oxidation/reduction reactions developing downgradient from a gasoline spill. Envision a deep, sandy sediment housing an underground storage tank used to dispense automobile fuel. The tank corrodes and gasoline spills into the subsurface – reaching the water table and dissolving in the aqueous phase. The prespill conditions of the groundwater show significant concentrations of O_2, NO_3^-, and sulfate. Furthermore, the sand grains are coated with both iron and manganese oxides. Gasoline is a complex carbon source (alkanes, aromatics, and other compounds; see Sections 7.3 and 8.3) whose components can be metabolized by a subset of populations originally residing in the subsurface microbial community. Immediately adjacent to the spill, microorganisms flourish using gasoline components as electron donors and oxygen as the electron acceptor. This zone of aerobic respiratory activity spreads downgradient with the gasoline moving in groundwater, leaving behind a zone of oxygen depletion. Then, nitrate-respiring microorganisms flourish in an expanding front until nitrate is exhausted. This process of electron-acceptor exhaustion continues through the thermodynamic hierarchy of free energy-yielding reactions shown in Figure 3.10 and Table 3.7 until CO_2 is reached. The bottom panel of Box 3.7 depicts the electron-accepting processes that characteristically occur downgradient in a mature, contaminated groundwater site. Closest to the contamination, methane can be found. Further downgradient, samples of water and/or sediment routinely reveal elevated concentrations of sulfide. Further along are zones rich in Fe^{2+} and Mn^{2+} and nitrite. Finally, far from the contamination, an unaffected aerobic zone can be found. The spatially distinctive zones of microbial physiological processes shown in Box 3.7 are striking evidence for the responsiveness of microorganisms to environmental perturbation (in this case, gasoline pollution) and for the geochemical impact of those responses. Simply by making a living as chemosynthetic organoheterotrophs, naturally occurring subsurface microorganisms consume (biodegrade) gasoline and drastically alter their geochemical setting. Most important: these alterations are predictable based on thermodynamic relationships shown in Figure 3.10.

Information presented in Table 3.7 is illustrative of crucial relationships between physiological energetics and biogeochemistry, but the list of microbial processes in Table 3.7 is not exhaustive. Additional microbially mediated biogeochemical processes are presented in Sections 3.9 to 3.11 and in Sections 7.3 to 7.5. A key concept that will be emphasized in Chapter 7 is that

Box 3.7

Field demonstration of the sequence of thermodynamically predicted physiological processes

See the text for an explanation of how sequential depletion of final electron acceptors leads to the zones of biogeochemical processes shown below.

material resources in the biosphere are both produced and consumed. The pathways of production and consumption are dependent upon a combination of the specific geochemical settings, thermodynamic instabilities, and the composition of ambient native microbial communities. Often multiple biogeochemical mechanisms have the potential to consume a particular substance in a given habitat. Consider the second entry in Table 3.7. Denitrification (conversion of nitrate (as a respiratory terminal electron acceptor) through a series of enzymatic reductions to N_2 gas) is a key pathway that consumes nitrate in anaerobic soils, sediments, and waters. However, several other microbial processes can anaerobically consume nitrate; these include dissimilatory reduction of nitrate to ammonia (DNRA) (via nitrite), anaerobic ammonia oxidation (anammox), and nitrate reduction tied to oxidation of both methane and elemental sulfur (see Section 3.11). Documenting the occurrence of particular pathways in real-world field sites and

understanding controls beyond thermodynamics that favor one process over another are among the frontiers of environmental microbiology research.

3.9 OVERVIEW OF METABOLISM AND THE "LOGIC OF ELECTRON TRANSPORT"

It is beneficial to place the concepts of "resource exploitation" and "oxidation/reduction reactions" within the overall physiological function of the microbial cell. We have emphasized in Sections 3.3 to 3.8 (especially Section 3.6) that ATP generation assists cellular processes by fueling cell maintenance and cell growth. As shown in Figure 3.11, cellular metabolism broadly consists of catabolism and anabolism. Catabolism is the cell's network of reactions for ATP generation. Anabolism is the cell's network of reactions, fueled by ATP, that assemble molecular building blocks (inorganic nutrients and low molecular weight organic compounds transported to the cell's interior) into new cell constituents or into progeny cells. "Habitat resources" in Figure 3.11 are the wide variety of organic and inorganic compounds that occur in water, sediments, and soils. These include electron donors that are thermodynamically unstable in the presence of electron acceptors. During catabolism by microorganisms endowed with electron transport chains, the electron acceptor terminates the series of electron transport reactions within the cytoplasmic membrane. For this reason, the term, *terminal electron acceptor*, is frequently used. After the electrons have been accepted, the reduced materials (e.g., H_2O, NO_2^-, N_2, NH_3, Mn^{2+}, Fe^{2+}, CH_4) are waste materials,

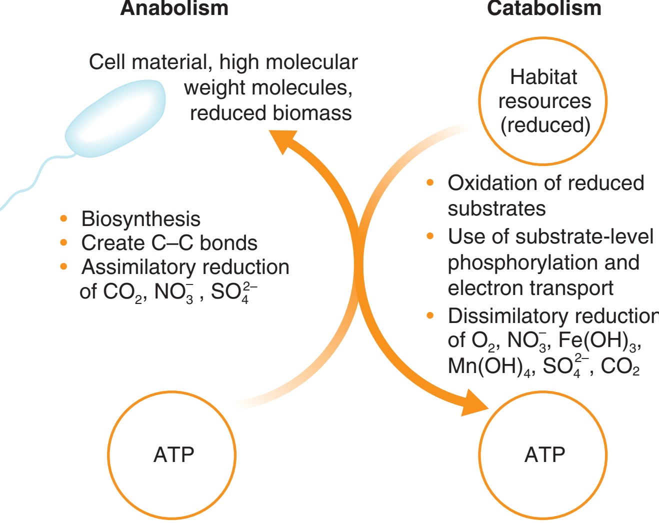


Figure 3.11 Overview of cellular metabolism: catabolism creates ATP, which fuels the biosynthetic reactions of anabolism.

discarded extracellularly; they are not assimilated into cell material. During anabolic reactions (Figure 3.11) biosynthetic reactions (driven by ATP) forge new bonds, assemble macromolecules, and ultimately lead to the assembly of new (progeny) cells. As cellular components are assembled, oxidized inorganic compounds (e.g., NO_3^-, SO_4^{2-}, CO_2) may be reduced – as required for their assimilation into new biomass. This anabolically driven reduction of inorganic compounds, termed "assimilatory reduction", consumes ATP and is distinctive from dissimilatory reduction carried out during catabolism.

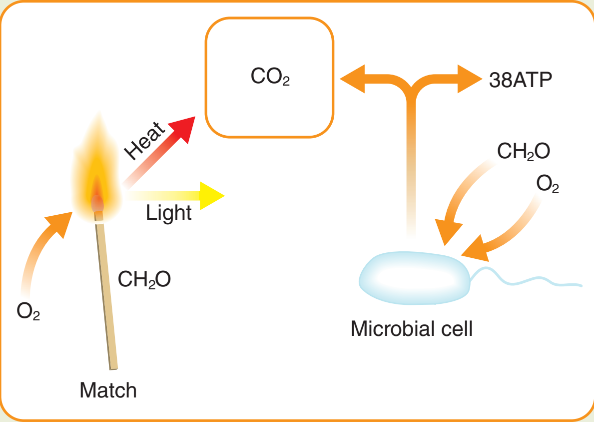
The lower panel in Box 3.8 provides an example of the biochemical manifestation of catabolism. During glycolysis (or the Embden–Meyerhoff–Parnas pathway), enzyme-mediated transformation of glucose (a six-carbon molecule) delivers two molecules of pyruvate (three carbons each) to the citric acid cycle. As electrons are removed from intermediates, CO_2 is produced and the electrons (as NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide phosphate)) are delivered to an electron transport chain driven by oxygen as the terminal electron acceptor. The result is 38 ATP molecules per molecule of glucose. Glucose, $C_6(H_2O)_6$, is thermodynamically unstable in the presence of oxygen. If glucose were to burn via a direct chemical reaction with oxygen, it would produce light and heat, much like a matchstick (upper panel, Box 3.8). The energy released (heat and light) when a match burns is the same energy released when an equivalent mass of glucose is oxidized by a microbial cell. The membrane architecture and electron transport systems that convert the free energy of combustion to biochemically useful ATP are one of the many impressive adaptations (miracles) of life. A key feature of the lower portion of Box 3.8 is exploitation of the electron transport chain. As long as there is an extracellular terminal electron acceptor available to consume electrons, and hence drive the flow of electrons through the electron transport chain, ATP production is assured. In Box 3.8, oxygen is the driver – but other compounds in the hierarchy in Figure 3.10 function similarly. In general, microorganisms carrying out catabolism use fine-tuned enzymatic pathways (analogous to the citric acid cycle) to oxidize reduced substrates (inorganic compounds if chemolithotrophs; organic compounds if chemoorganotrophs) to deliver electrons to the respiratory chains of electron transport systems. Aerobic microorganisms utilizing glycolysis and the citric acid cycle have a high yield of ATP. If oxygen is unavailable, another biochemical mechanism (encoded by a corresponding set of genes) may be expressed and enable the cell to use other terminal electron acceptors. The thermodynamic hierarchy (see Table 3.7 and Figure 3.10) shows the energetics of the reactions.

3.10 THE FLOW OF CARBON AND ELECTRONS IN ANAEROBIC FOOD CHAINS: SYNTROPHY IS THE RULE

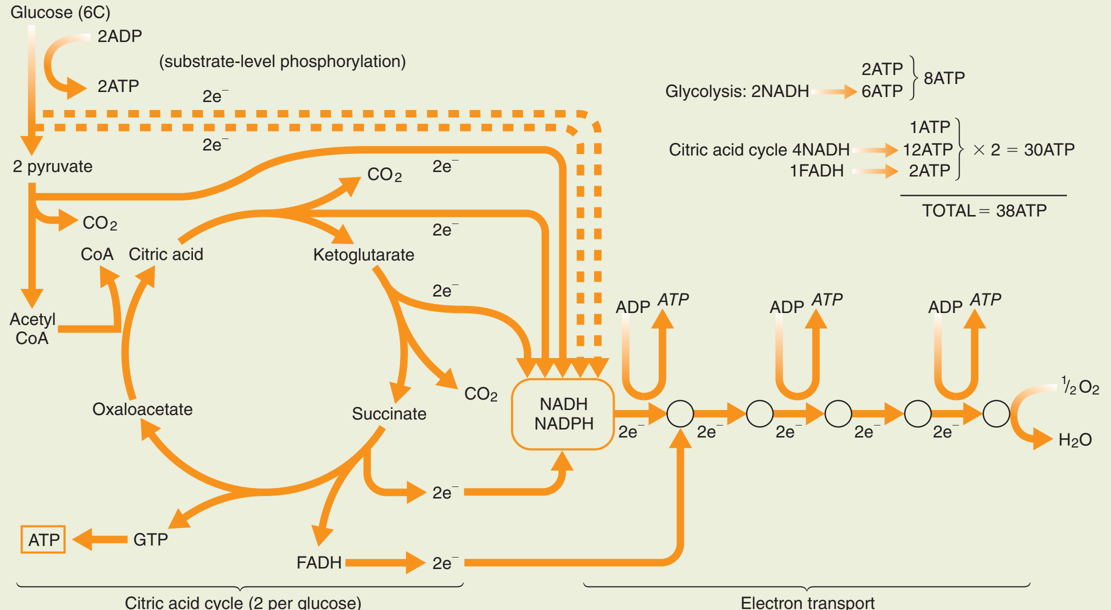
In Section 3.9 and Box 3.8, we saw that glycolysis, the citric acid cycle, and the electron transport chain allow aerobic microorganisms to exploit a

Box 3.8

The miracle of electron transport for ATP production: comparing a burning match to aerobic respiration



Summary of glycolysis, the citric acid cycle, and aerobic respiration



Each of the five circles in the Electron Transport Chain represents a membrane-associated electron carrier (e.g., cytochrome complex) that generates proton-motive force, hence ATP, via ATP synthase.

prevalent resource in the biosphere: glucose. The energetics of aerobic respiration (see Table 3.7 and Figure 3.10) predict that it will happen. However, only the tools of physiology, biochemistry, and genetics applied to a particular model bacterium can reveal details of the mechanisms. After such tools have been applied to many modes of catabolism carried out by many model microorganisms, clear themes and patterns emerge (see Section 7.5). These themes constitute an established body of physiological knowledge codified as mechanisms often named for their discoverers, such as the Embden–Meyerhoff–Parnas pathway, the Entner–Douderoff pathway, the Wood–Ljungdahl pathway, the Krebs tricarboxylic acid cycle, and the Calvin cycle (Kluyver and van Niel, 1954; Gottschalk, 1986; Zehnder and Stumm, 1988; Niedhardt et al., 1990; Ljungdahl et al., 2010; Ferry, 1993; Drake, 1994; White et al., 2012; Lengeler et al., 1999; Wackett and Hershberger, 2001; Drake et al., 2006).

Many of the processes, especially anaerobic ones, depicted by simple equations in Table 3.7 cannot be catalyzed by pure cultures of single microorganisms. Instead, they are only carried out by cooperating populations of physiologically distinctive microorganisms. These *syntrophic associations of microorganisms* are often critical for organic carbon-driven iron reduction, manganese reduction, sulfate reduction, acetogenesis, and methanogenesis (lower group of entries in Table 3.7). In syntrophic associations, an anaerobic food web is established in which metabolic byproducts of one group of microorganisms are essential substrates for another (Sieber et al., 2012). Figure 3.12 features the flow of carbon electrons and energy among cooperating populations of anaerobic microorganisms. Typical organic carbon materials reaching soils, lakes, sediments, and sewage treatment plants are high molecular weight polymers such as cellulose. Fermentative microorganisms hydrolyze the polymers into low molecular weight constituents that undergo fermentation reactions. Fermentation, by definition, represents no net change in oxidation–reduction status of the substrate. In chemical terms, fermentations are disproportionation reactions in which a portion of the organic substrate pool is oxidized to CO_2 while the remainder is reduced as an electron acceptor. The reduced waste products of fermentation include fatty acids (e.g., lactate, succinate, butyrate, acetate), alcohols, and hydrogen gas. These pools of fermentation waste products are valuable metabolic resources for the various metabolic groups known as iron reducers, manganese reducers, sulfate reducers, acetogens, and methanogens. Hydrogen (H_2) plays a particularly critical role in these interacting populations because it is an important electron donor used by most of these groups (see Section 3.8). Figure 3.12b depicts anaerobic food webs as two-step processes driven by populations responsible for the second step. Many of the fermentation reactions that occur in

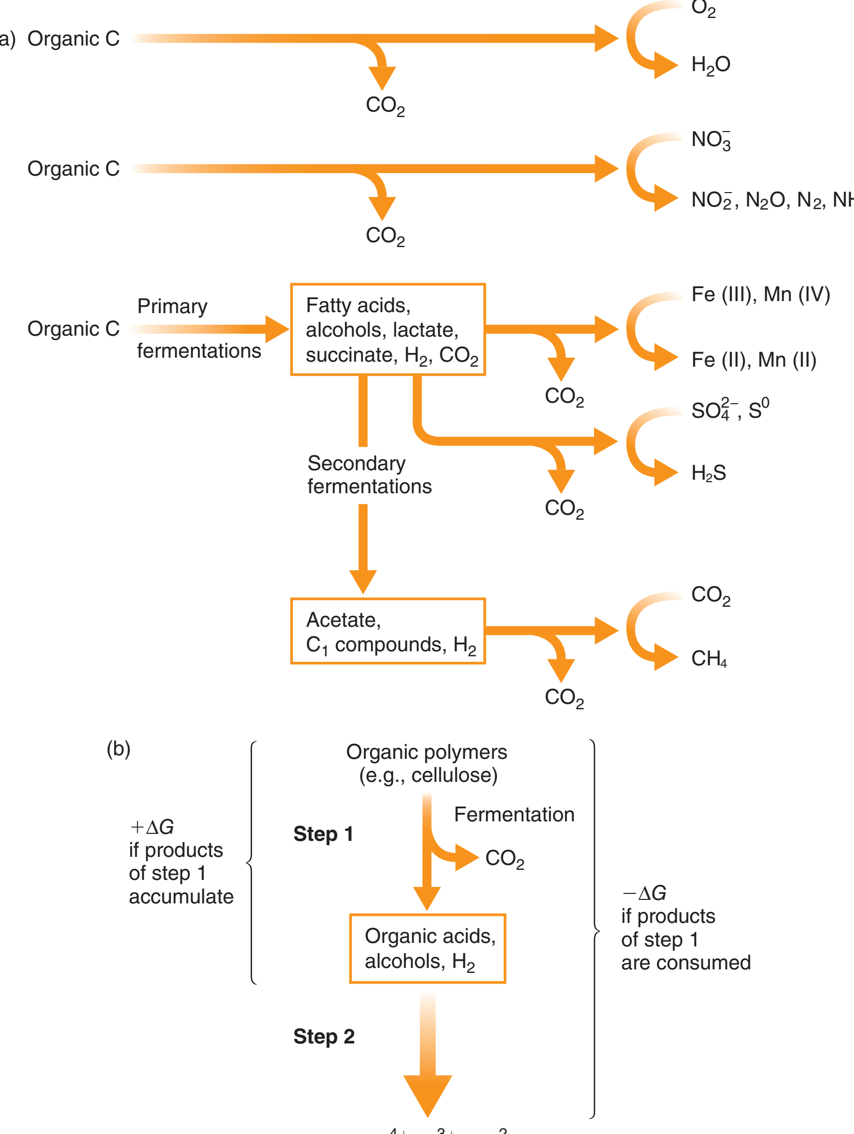


Figure 3.12 Flow of carbon and electrons in anaerobic food chains. (a) Contrasts between oxygen and nitrate respiration (carried out by individual microorganisms) and the cooperation of multiple populations of anaerobes that occur during iron reduction, manganese reduction, sulfate reduction, and methanogenesis. (b) How metabolic scavenging (step 2) allows anaerobic processes to overcome potential thermodynamic barriers. ((a) From Lengeler, J.W., G. Drews, and H.G. Schlegel (eds). 1999. *Biology of Prokaryotes*. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

nature are not energetically favorable (+Δ*G*) if the endproducts are allowed to accumulate. However, because populations involved in the second step are effective at scavenging the fermentation products, especially H_2, the overall process becomes thermodynamically favorable. Thus, syntrophy (the metabolic cooperation between distinctive populations) is crucial for the success of many anaerobic processes. The term *interspecies hydrogen transfer* has been coined in recognition of hydrogen's key role in this cooperative metabolism. Note that a major methanogenesis pathway (in addition to CO_2 reduction by H_2) is fermentation of acetate and methyl-containing compounds: acetoclastic methanogenesis.

3.11 THE DIVERSITY OF LITHOTROPHIC REACTIONS

The perceptive reader may have realized that the biogeochemical compass depicted in Figure 3.10 applies to organic and inorganic compounds equally well. To chart potential biogeochemical reactions between inorganic electron donors in Figure 3.10 (e.g., H_2S, H_2, NH_3, CH_4), one only needs to draw arrows connecting reduced substances on the right of the half reaction scale to oxidized substances on the left (see also Box 3.4). For example, hydrogen-metabolizing microorganisms can link hydrogen oxidation to oxygen reduction (a long arrow), nitrate reduction (a slightly shorter arrow), sulfate reduction (a short arrow), or methanogenesis (the shortest arrow). Any reduced substrate (lower right on the half-reaction scale) can be linked to any oxidized substrate (upper left on the half-reaction hierarchy scale). All combinations of electron-donor–electron-acceptor reactions predicted by thermodynamics may not yet have been documented as genuine biochemical processes in microorganisms. Two physiological processes, anaerobic oxidation of methane and ammonium, were suspected to be important biogeochemical process in aquatic habitats for decades – but have only been well documented, microbiologically, since the 1990s (see Sections 7.4 and 7.5). Another related physiological process, the use of methane as an electron donor and nitrate/nitrite as electron acceptors, was discovered in 2006 (Raghoebarsing et al., 2006) and later found to be reliant upon a completely novel, oxygen-generating intracellular enzymatic reaction in 2010 (Ettwig et al., 2010, 2012; see Sections 7.3 to 7.5 and Box 7.10). Table 3.8 provides a summary of 28 established chemolithotrophic reactions used by microorganisms. Strictly speaking, microbial methane oxidation falls into its own separate category of physiological reactions, known as "C_1 metabolism" (Madigan et al., 2014; White et al., 2012). As illustrated for chemosynthetic organotrophs (see Sections 3.9 and 3.10), the thermodynamic compass reveals energetic impetus for catabolic reactions, not the biochemical mechanisms nor the genes that underlie them. Details of the biochemistry and genetics of chemolithoautotrophy are current areas of active research.

Table 3.8

Well-characterized chemolithotrophic and methanotrophic reactions and their respective energy and growth yields. (Modified from Kelly, D.P. and A.P. Wood. 2013. The chemolithotrophic prokaryotes. In: M.W. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (eds), *The Prokaryotes*, Vol. 2, 3rd edn, pp. 441–456. Springer-Verlag, New York. With kind permission of Springer Science and Business Media.)

Reaction	Substrate oxidized	$\Delta G°$ (kJ/mol substrate)	Estimated number of mol ATP synthesized/ mol substrate
$H_2 + 0.5O_2 \rightarrow H_2O$	H_2	−237	2–3
$5H_2 + 2NO_3^- + 2H^+ \rightarrow N_2 + 6H_2O$	H_2	−241	
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	H_2	−35	<0.25?
$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$	NH_4^+	−272	1 or 2
$NH_4^+ + 0.75O_2 \rightarrow 0.5\ N_2 + 1.5\ H_2O + H^+$	NH_4^+	−315	
$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	NH_4^+	−361	
$NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$	NH_2OH	−288	2
$NO_2^- + 0.5O_2 \rightarrow NO_3^-$	NO_2^-	−73	1
$H_2S + 0.5O_2 \rightarrow S^0 + H_2O$	H_2S	−209	1?
$S^0 + 1.5O_2 + H_2O \rightarrow H_2SO_4$	S^0	−519	1–3?
$S^0 + 6/5\ NO_3^- + 2/5\ H_2O \rightarrow SO_4^{2-} + 3/5\ N^2 + 4/5\ H^+$	S^0	+515	
$S^0 + 3\ NO_3^- + H_2O \rightarrow 3\ NO_2^- + SO_4^{2-} + 2H^+$	S^0	−352	
$S^0 + 6Fe^{3+} + 4\ H_2O \rightarrow HSO_4^- + 6Fe^{2+} + 7H^+$	S^0	−314	
$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+ + 7H^+$	HS^-	−733	1.5–4?
$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$	$S_2O_3^{2-}$	−739	2.3
$5S_2O_3^{2-} + 8\ NO_3^- + H_2O \rightarrow 10\ SO_4^{2-} + 2H^+ + 4N_2$	$S_2O_3^{2-}$	−751	4–5
$S_4O_6^{2-} + 3.5O_2 + 3H_2O \rightarrow 4\ SO_4^{2-} + 6H^+$	$S_4O_6^{2-}$	−1245	5
$5\ S_4O_6^{2-} + 14NO_3^- + 8H_2O \rightarrow 20\ SO_4^{2-} + 16H^+ + 7N_2$	$S_4O_6^{2-}$	−1266	8–10
$2Fe^{2+} + 2H^+ + 0.5O_2 \rightarrow 2Fe^{3+} + H_2O$	Fe^{2+}	−47	0.5
$4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$	FeS_2	−1210	
$Cu_2S + 0.5O_2 + H_2SO_4 \rightarrow CuS + CuSO_4 + H_2O$ (oxidation of Cu^+ to Cu^{2+})	Cu_2S	−120	1?
$CuSe + 0.5O_2 + H_2SO_4 \rightarrow CuSO_4 + Se^0 + H_2O$ (oxidation of selenide to selenium)	CuSe	−124	1?
$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	CH_4	−871	
$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$	CH_4	−556	
$CH_4 + 8/3\ NO_2^- + 8/3H^+ \rightarrow CO_2 + 4/3N_2 + 10/3H_2O$	CH_4	−309	
$CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O$	CH_4	−270	
$CH_4 + 8/5\ NO_3^- + 8/5H^+ \rightarrow CO_2 + 4/5N_2 + 14/5H_2O$	CH_4	−153	
$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + H_2S^- + H_2O$	CH_4	−20 to −40	0.5?

STUDY QUESTIONS

1 Regarding trends in genomics, how would you interpret discovery of a microorganism with a large genome that dwells intracellularly?

2 Unknown hypothetical genes constitute roughly one-third of all known genomes. As a curious microbiologist, you want to discover if a portion of these genes are still useful to their hosts. Describe experiments aimed at assessing when and if unknown hypothetical genes become active in their hosts in nature.

3 In the carbon–energy matrix of Table 3.3, one combination is clearly absent: chemosynthetic organoautotrophs. Can you suggest a reason why this "theoretically possible" class of physiology does not seem to exist?

4 Table 3.5 briefly describes a variety of genetic systems that govern microbial responses to nutrient stress. The fourth entry lists how ammonia limitation activates nitrogen fixation (*nif*) genes. Explain the physiological benefit of this regulatory circuit.

5 In Section 3.5, a quote appears from J. Poindexter, "over extended periods of time, conservative utilization of nutrients once they are in the cell . . . may be more important than high-affinity uptake systems."
   (A) Do you agree with this statement? Why or Why not?
   (B) Under what circumstances is the statement likely to be true? Under what circumstances is it likely to be false?
   (Hint: in preparing your answers, consider data in Table 3.6.)

6 Calculate the free energy yield available to two types of nitrate-reducing microorganisms – those converting nitrate to nitrogen gas (N_2; e.g., *Pseudomonas*) and those converting nitrate to ammonia (*E. coli*). First, write the two reactions and balance them. Assume that the electron donor for each reaction is carbohydrate (CH_2O). To carry out the calculation, use Box 3.4 (see the final preferred approach for calculating the biochemical free energy yield) and Figure 3.10. Assume E_o' values for the electron donor and two electron acceptors are as follows: CO_2/CH_2O, −0.43 V; NO_3^-/NH_3, +0.36 V; NO_3^-/N_2, +0.75 V.

7 Regarding your answer to question 6, given the significantly smaller free energy yield when nitrate is reduced to ammonia, why would *E. coli* use dissimilatory reduction of nitrate to ammonia? (Hint: consider the stoichiometry, especially the number of electrons accepted. For additional background, see Section 7.4.)

8 Note that the stoichiometric redox equations on the right-hand side of Table 3.7 are written with carbohydrate (CH_2O) as the electron donor.
   (A) Prepare balanced redox equations for metabolism of the fuel component, toluene (C_7H_8), under aerobic, denitrifying, iron-reducing, sulfate-reducing, and methanogenic conditions. Be sure to account first for the electrons transferred in the redox reactions, then use mass, charge, water, and protons to complete the balancing (see Box 3.4).
   (B) In both sets of equations (glucose and toluene) some of these metabolic processes consume H^+. What geochemical impact would this have on field sites where these processes occur?

9 A deep-sea hydrothermal vent emits dissolved concentrations of hydrogen gas, methane, hydrogen sulfide, and Fe^{2+} into aerobic waters.
   (A) Write balanced stoichiometric reactions between oxygen (electron acceptor) and each of the four electron donors.
   (B) Using the thermodynamic tools explained in Box 3.4 and Figure 3.10, calculate the free energy yield for each reaction to rank the four potential electron donors from the most to the least physiologically beneficial. Assume (as given in Box 3.4 and/or can be read from

Figure 3.10) that the E_o' values for O_2/H_2O, Fe^{3+}/Fe^{2+}, SO_4^{2-}/H_2S, CO_2/CH_4, and H^+/H_2 are 0.82, 0.0, –0.19, –0.25, and –0.41, respectively.

(C) Regarding the microbiology of oceanic hydrothermal vent sites, what major factors other than those treated in (B) above are likely to determine processes and populations likely to flourish in vent communities?

(D) How would you prove that each of these potentially useful energy sources was actually used by microorganisms in situ?

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4

A Survey of the Earth's Microbial Habitats

Chapter 3 began with the concept that Earth habitats and life have been coevolving for 3.8×10^9 years. Furthermore, the coevolution can be metaphorically viewed as a dialog depicted as a double-headed arrow extending between the two spheres in Figure 3.1. The figure's left-hand sphere, labeled "Earth habitats", is the subject of this chapter. To set the stage for this chapter, you will need to recall the following points:

- *Some of the key physical events in planetary history include cooling from >100°C, alterations in the concentration of many atmospheric gases, meteor impacts, glaciation, volcanic activity, and plate tectonics (see Section 2.1 and Table 2.1).*
- *The histories of these physical planetary changes have been revealed by details in the* geologic record *(see Section 2.1).*
- *Key clues that have partially unlocked the history of the planetary* biological record *include fossils, ancient biomarkers, and stable isotopic ratios (see Section 2.1 and Table 2.2).*
- *The notion (the hypothesis) that genomes of extant microorganisms contain a* genetic record *of evolutionarily important selective pressures was presented in Section 3.2.*
- *In order to implement the prime directive (survive, maintain, grow: see Sections 1.1 and 3.4), all life forms must exploit planetary resources – especially those manifest as carbon and energy sources (see Section 3.3).*

Chapter 4 Outline

4.1 Terrestrial biomes
4.2 Soils: geographic features relevant to both vegetation and microorganisms
4.3 Aquatic habitats
4.4 Subsurface habitats: oceanic and terrestrial
4.5 Defining the prokaryotic biosphere: where do prokaryotes occur on Earth?
4.6 Life at the micron scale: an excursion into the microhabitat of soil microorganisms
4.7 Extreme habitats for life and microbiological adaptations

However, the diverse habitats of Earth present many selective pressures in addition *to carbon and energy sources. This chapter will take the reader on a tour of terrestrial and aquatic habitats that currently exist on Earth. After a survey of habitat characteristics (including prokaryotic biomass itself), we will focus on extremes of environmental conditions that have confronted microorganisms and explore the adaptive biochemical mechanisms that have resulted.*

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4.1 TERRESTRIAL BIOMES

Major climatic determinants for world biomes are temperature (a function of both elevation and latitude) and atmospheric precipitation. Figure 4.1 depicts the present-day distribution of vegetation-defined biomes across the globe. The 12 major biome types are: tundra, boreal forest, temperate deciduous forest, temperate grassland, dry woodland/shrubland (chaparral), desert, tropical rain forest/evergreen forest, tropical deciduous forest, tropical scrub forest, tropical savanna thorn forest, semidesert arid grassland, and mountains (complex biome zonation). Several striking patterns appear in Figure 4.1. Tundra predominates in circumpolar regions of the

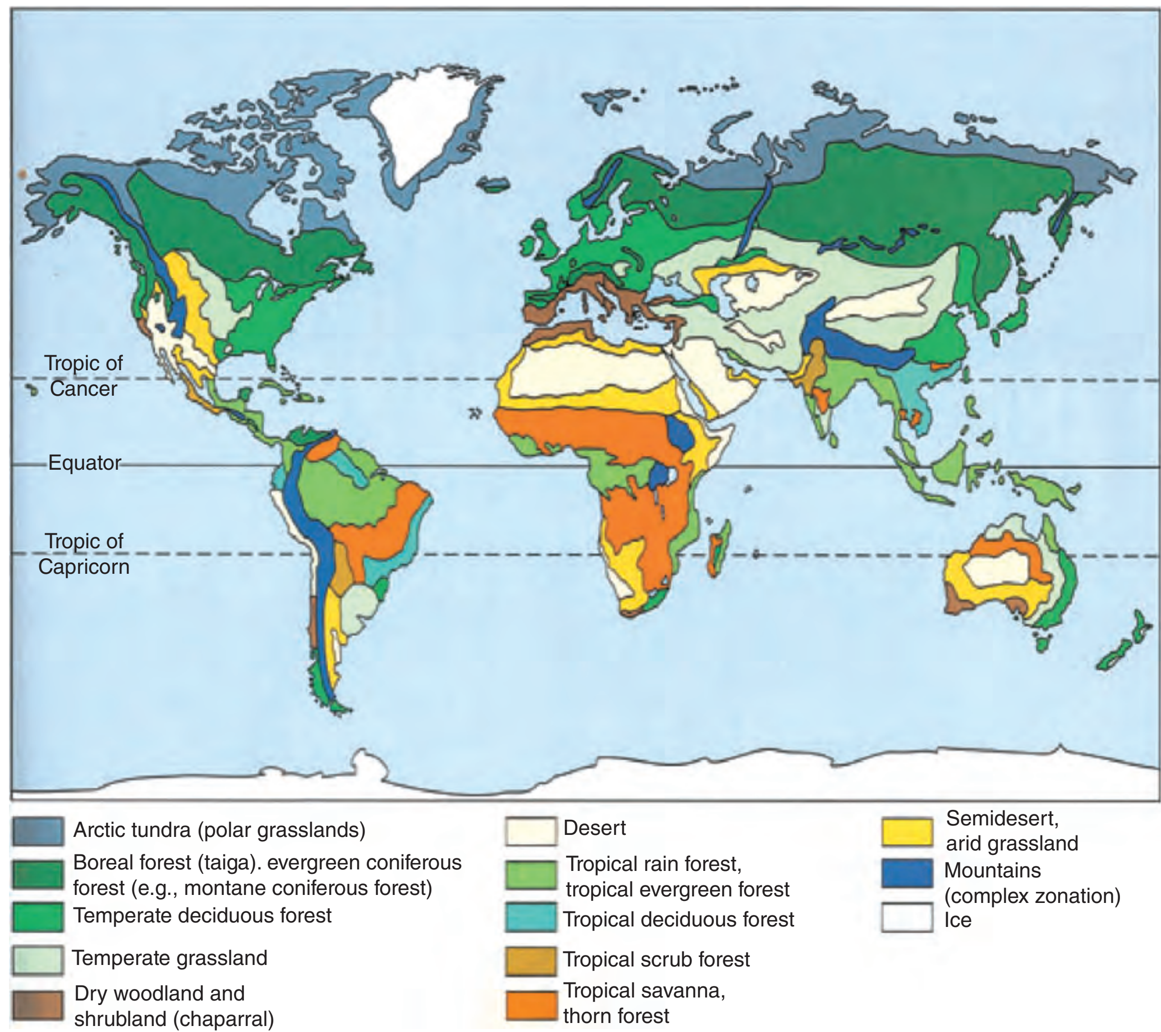


Figure 4.1 Global map of terrestrial biomes. (Republished with permission of Brooks/Cole, a division of Thomson Learning. From Miller, G.T. 2004. *Living in the Environment*, 13th edn. Permission conveyed through Copyright Clearance Center, Inc.)

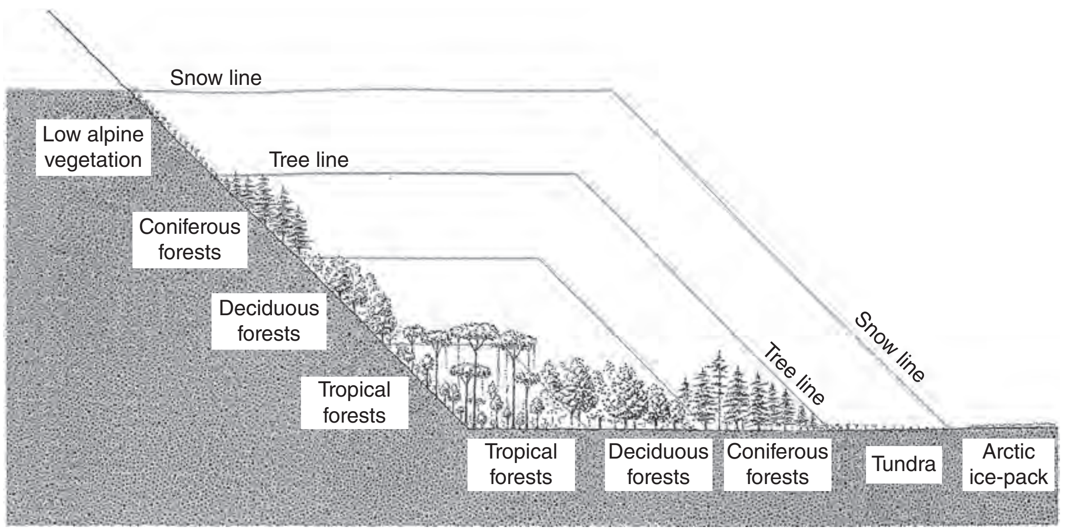


Figure 4.2 Examples of regional and altitudinal gradients of vegetation zones in North America. The south–north gradient (right side of diagram, horizontal line) primarily reflects gradually cooling temperature regimes that extend from the hot tropics (low latitudes) to the frigid arctic (high latitudes). In parallel with the south–north gradient, many mountain ranges are hosts of similar vegetation zones that change with elevation (altitude; left side of diagram, diagonal line). (Source: Colinvaux, P.A. 1973. *Introduction to Ecology.* John Wiley and Sons, Inc., New York. Figure 2.5, page 28. Reprinted with permission.)

northern hemisphere. There is a broad band of boreal forest south of the tundra in the northern hemisphere and this blends into temperate deciduous forest and/or temperate grassland steppe at mid-latitudes in both hemispheres. Tropical savanna–thorn forest biomes occupy significant portions of northern Australia, Southeast Asia, India, the Arabian Peninsula, Africa, and South America. Tropical forests occupy broad equatorial swaths of the globe – across Central and South America, Central Africa, Southeast Asia, and the Malay Archipelago. Semidesert arid grasslands occur in subequatorial South America, equatorial Africa, and Australia. Deserts occur globally at mid-latitudes in southwestern North America, South Africa, Central Australia, and from northern Africa, eastward beyond the Arabian Peninsula.

Examples of regional and elevational vegetation-based biome gradients are depicted in Figure 4.2. A north–south transect in North America extends from tundra (extreme right side of Figure 4.2), through boreal coniferous forest, to the mixed mesophytic forests of the Appalachian Mountains, to subtropical forests of Florida and Mexico, on to the tropical forests of southern Mexico (center of Figure 4.2). These regional north–south patterns in naturally occurring plant communities (and habitat conditions that govern them) are mirror images of patterns found on slopes of tropical mountains over a range of elevations (Figure 4.3, center to left). East–west patterns in habitat conditions can also strongly influence patterns of naturally occurring plant biomes. For

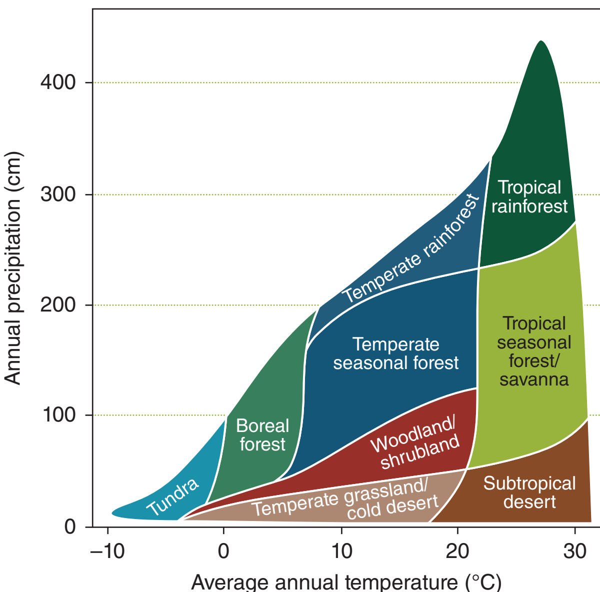


Figure 4.3 The pattern of plant biome types showing responses to annual habitat precipitation (vertical axis) and annual temperature (horizontal axis). Boundaries between the nine plant biome types are approximate– influenced by factors that include soil type, maritime versus continental climate, and fire. (Source: "PrecipitationTempBiomes". Via Wikipedia: http://en.wikipedia.org/wiki/File:PrecipitationTempBiomes.jpg#mediaviewer/File:PrecipitationTempBiomes.jpg. After R.D. Burkett, posted to the Wikimedia Commons, based on Whittaker, R.H. 1975. *Communities and Ecosystems*, 2nd edn. Macmillan Publishing Co. Inc., New York.)

example, holding latitude roughly constant but varying precipitation, a hiker moving west from the forest in the Appalachian Mountains in North America will pass through mixed mesophytic forest (moist broad-leaved species including maple, beech, birch, ash, and rhododendron), oak–hickory forest, bur oak, and grasslands to prairie, then short-grass plains and on to the desert.

The global distributions of plant-biome types described above have long been recognized by ecologists to be manifestations of physiological adaptations by plants to two major environmental determinants: water availability and temperature. Figure 4.3 shows how relationships between average annual precipitation and temperature can be used to predict where a given plant-biome type will occur on Earth.

4.2 SOILS: GEOGRAPHIC FEATURES RELEVANT TO BOTH VEGETATION AND MICROORGANISMS

Soils are vital for the biosphere – serving as the "skin of the Earth" in terrestrial habitats. Soils are the medium for root growth and nutrient uptake by plants. Soils are a major site for nutrient cycling (see Sections 7.3 and 7.4) – acting as reservoir for both plant-derived nutrients and for vast microbial diversity. At any given location on the Earth's terrestrial surface, the type of soil that develops reflects a combination of five factors: geologically derived parent material, climate, vegetation, time, and topography (Brady and Weil, 2007; Gardiner and Miller, 2004). The soil matrix is a three-dimensional porous array consisting of inorganic solids (sand, silt, and clay) intermingled with deceased biomass, humic materials, and organic and inorganic chemical coatings (humus and amorphous oxides), as well as viable organisms (micro- and meso-flora and fauna, especially fungi, protozoa, insects, nematodes, and burrowing animals). The pore spaces are shared in variable proportions by gases (whose composition reflects a balance between atmospheric diffusion and biotic activity) and aqueous soil solution (whose composition reflects complex equilibria between inorganic, organic, and biotic reactions; Madsen, 1996).

The global geography of specific soil types and the processes that contribute to their formation have been well studied. Table 4.1 extends the relationships

Table 4.1

Relationship between global regions, climate types, biomes, and their associated soil orders. (Compiled from Smith, 1990, Palm et al., 2007, and Legros, 2013)

Region	Climate type	Biome type/vegetation	Dominant soil orders (areal distribution)
Polar	Tundra	Tundra	Gelisols (79%), inceptisols (16%)
	Subarctic	Boreal forest/Taiga	Inceptisol (36%), gelisol (25%), spodosol (19%), alfisol (9%), histosol (6%)
Temperate	Continental	Coniferous forest	Inceptisol (33%), alfisol (12%), ultisol (12%), mollisol (10%), spodosol (9%)
	Continental	Broadleaf deciduous and mixed forest	Inceptisol (30%), alfisol (24%), entisol (12%), mollisol (10%), ultisol (9%)
	Continental	Grassland, savanna, and shrubland	Mollisol (53%), aridisol (18%), entisol (12%)
	Wet	Flooded grassland and savanna	Alfisol (32%), entisol (19%), vertisol (14%), aridisol (10%)
	Mediterranean	Mediterranean forest, woodland, and shrub	Inceptisol (25%), alfisol (24%), entisol (23%), aridisol (17%)
	Desert	Deserts and xeric shrubland	Aridisol (45%), entisol (43%)
Tropical/subtropical	Continental	Coniferous forest	Inceptisol (25%), alfisol (21%), mollisol (15%), andisol (12%)
	Continental	Grassland, savanna, shrubland	Entisol (32%), alfisol (17%), oxisol (17%), ultisol (12%)
	Continental	Dry broadleaf forest	Alfisol (24%), inceptisol (16%), entisol (15%), ultisol (14%), vertisol (12%)
	Continental	Moist broadleaf forest	Oxisol (32%), ultisol (30%), inceptisol (17%)
Montane	Continental	Grassland and shrubland	Gelisol (35%), inceptisol (27%), entisol (13%), aridisol (9%)

between climate and biome type to the associated soil types. In ice-free polar zones (mean temperature of the warmest month <10°C), where tundra predominates and many soils remain frozen within the top 200 cm, gelisols ("gel" for gel-like) occur. In these polar (and some montane and some recently eroded) regions, the underlying geologic parent material has undergone minimal soil development; these soils are known as entisols ("ent" for recent) and inceptisols ("incept" for inception, indicating a young embryonic stage with little or no horizon development). Table 4.2 provides a summary of the 12 global soil orders developed for the United States soil taxonomy system. In subarctic polar zones (mean temperature of summer is 10°C; of winter, −3°C), inceptisols, gelisols, spodosols, and histosols can be encountered (Table 4.1).

Table 4.2

Names of the 12 soil orders of the world, as specified in the US soil taxonomy, with their major characteristics. (From Gardiner, D.T. and R.W. Miller. 2004. *Soils in Our Environment*, 10th edn, p. 205. Pearson/Prentice Hall, Englewood Cliffs, NJ. Copyright 2004, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ)

Soil order*	General features
Gelisols	Gelisols have permafrost within 200 cm of the surface
Entisols	Entisols have no profile development except perhaps a shallow marginal A horizon. Many recent river flood plains, volcanic ash deposits, unconsolidated deposits with horizons eroded away, and sands are entisols
Inceptisols	Inceptisols, especially in humid regions, have weak to moderate horizon development. Horizon development is minimal because of cold climates, waterlogged soils, or lack of time for stronger development
Andisols	Andisols are soils with more than 60% volcanic ejecta (ash, cinders, pumice, basalt) with bulk densities below 900 kg/m^3. They have enough weathering to produce dark A horizons and early-stage amorphous clays. Andisols have high adsorption and immobilization of phosphorus and very high cation exchange capacities
Histosols	Histosols are organic soils (peats and mucks) consisting of variable depths of accumulated plant remains in bogs, marshes, and swamps
Aridisols	Aridisols exist in dry climates, and some have developed horizons of lime or gypsum accumulations, salty layers and/or A and clay-rich Bt horizons
Mollisols	Mollisols are mostly in grasslands below some broadleaf forest-covered soils with relatively deep, dark A horizons; they often have B horizons with lime accumulations
Vertisols	Vertisols have high contents of clays that swell when wetted. A vertisol requires distinct wet and dry seasons to develop because deep wide cracks when the soil is dry are a necessary feature. Usually, vertisols have deep self-mixed A horizons (topsoil falls into cracks seasonally, gradually mixing the soil to the depth of the cracking). These soils exist mostly in temperate to tropical climates with distinct wet and dry seasons
Alfisols	Alfisols develop in humid and subhumid climates, have precipitation of 500–1300 mm, and are frequently under forest vegetation. Clay accumulation in a Bt horizon and available water much of the growing season are characteristic features. A thick E horizon is also common. They are slightly to moderately acidic
Spodosols	Spodosols are typically the sandy, leached soils of coniferous forests. Usually organic surface O horizons, strongly acidic profiles, and well-leached E horizons are expected. The most characteristic feature is a Bh or Bs horizon with accumulated organic material plus iron and aluminum oxides
Ultisols	Ultisols are strongly acidic, extensively weathered soils of tropical and subtropical climates. A thick E horizon and clay accumulation in a clay-rich Bt horizon are the most characteristic features
Oxisols	Oxisols are excessively weathered; few original minerals are left unweathered. Often, oxisols are more than 3 m deep, have low fertility, have dominantly iron and aluminum oxide clays, and are acidic. Oxisols develop only in tropical and subtropical climates

*Orders are arranged in approximate sequence from undeveloped soil to increased extent of profile development or increased extent of mineral weathering.

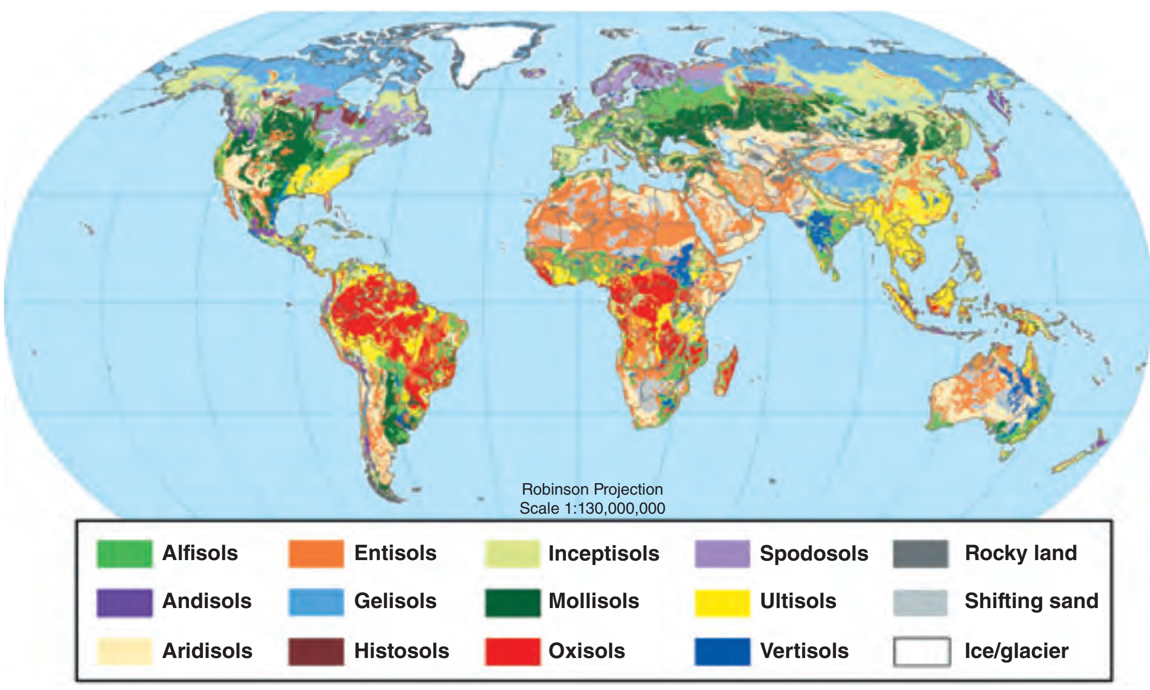


Figure 4.4 Distribution of 12 major soil orders throughout the world. (Reprinted with permission from USDA Natural Resources Conservation Service, Soil Survey Division, World Soil Resources, Washington, DC.)

Spodosols typically develop in both subpolar and temperate climates beneath coniferous forests where the combination of acidity, humus, and infiltrating water form a gray-colored spodic soil horizon rich in aluminum and iron oxides. Histosols ("hist" for tissue as in histology) occur in peat lands and bogs and are >20% organic matter. In humid temperate zones beneath mixed conifer–deciduous forests, a zone high in clay (known as an argillic horizon) often develops: this is a key characteristic of alfisols. Beneath temperate grassland (or "prairie") vegetation in humid temperate climates (Tables 4.1 and 4.2), the soil horizons are often dark and deep – fertile for agriculture and easy to work with a plow. These, known as mollisols ("moll" for mollify or soft), sometimes extend into dryer, short grass prairies, known as steppes. In the dry, warm conditions found in desert climates, only shrubs and sparse grasses contribute to the geochemical reactions governing soil formation. In this context aridisols develop. In the humid tropics, extremely weathered geologic strata occur at the Earth's surface. The heat, leaching from rainfall, and decomposing vegetation lead to the formation of highly oxidized minerals of iron and aluminum that are characteristic of oxisols (Table 4.2). Figure 4.4 shows the distribution of the major members of the 12 major soil orders throughout the continents. Compare Figures 4.3 and 4.1 and note how vegetation biomes and soil types coincide.

One of the key characteristics of soil habitats is that they feature an astonishing physical, chemical, and biological heterogeneity – in space and time,

and both locally and globally (see Section 4.6). All soils serve as depositories for deceased plant, animal, and microbial biomass. Unlike many aquatic habitats, there are few physical mechanisms for flushing materials through soil. What is deposited on the soil surface (e.g., plant material, deceased biomass, or microbial cells) generally remains there – serving as fodder for nutrient cycling and decay processes mediated by microorganisms (see Sections 7.3 to 7.6).

4.3 AQUATIC HABITATS

The Earth is the "blue planet": 70.8% of the surface is covered with water. Of the estimated 1.385×10^9 km^3 of water on the globe, 1.7% is ice pack (or glaciers), 96.5% is seawater, 1.7% is in groundwater, and only 0.014% is freshwater (in lakes, streams, and wetlands; Oki and Kenae, 2006). Thus, despite their crucial role for maintaining plants, animals, and humans on land, freshwaters comprise only a small proportion of the global water resources (Wetzel, 2001; Oki and Kenae, 2006). Table 4.3 compares several key characteristics of marine, freshwater, and groundwater reservoirs on Earth. Because of its huge volume and relatively small fluxes of incoming and outgoing waters, the residence time for water in the oceans is long (~3100 years). In contrast, the residence time of water in lakes, ponds, and rivers is relatively brief (~2 weeks to 10 years). After infiltrating through subsoil and entering the subsurface, the incoming water may re-emerge in surface habitats within ~2 weeks or may be trapped in deep aquifers for as long as 10,000 years. As shown in Table 4.3, subsurface habitats are quite distinctive (chemically and biologically) from open waters – largely due to the opaque, interstitial nature of rock and sediment matrices that influence geochemical reactions and exclude both large biota and light.

Freshwaters

Major freshwater resources of the globe include glaciers in polar and/or high elevation zones (North America, South America, Europe, Asia, Antarctica, Greenland), aquifers, and lakes. According to Wetzel (2001), inland waters cover less than 2% of the Earth's surface and only about 20 lakes are extremely deep – in excess of 400 m. Lake Baikal in Siberian Russia (with a surface area of 31,500 km^2, an average depth of 740 m, and a maximum depth of 1620 m) is by far the largest freshwater body in terms of volume (23,000 km^3). Wetzel (2001) has presented a diagram comparing the world's largest lakes on an areal basis (Figure 4.5). The Mediterranean Sea has a connection to the Black Sea; thus, it is not truly an inland lake. The Caspian Sea (436,400 km^2), though saline, is the largest lake. The Laurentian Great Lakes of North America (Lakes Superior, Huron, Michigan, Ontario, and Erie) constitute the greatest continuous mass of freshwater on Earth (245,240 km^2, with a collective volume of 24,620 km^3). In Africa, the largest freshwater body is Lake Victoria (68,870 km^2, with a volume of 2760 km^3) – the source of the White Nile. In South America, the largest freshwater body is Lake Titicaca (8372 km^2, with a volume of 893 km^3).

Table 4.3

Comparison between characteristics of three different aquatic habitats: marine, freshwater, and groundwater. (From Oki and Kenae, 2006, and Wetzel, 2001; and Madsen, E.L. and W.C. Ghiorse. 1993. Ground water microbiology: subsurface ecosystem processes. In: T. Ford (ed.), *Aquatic Microbiology: An Ecological Approach*, pp. 167–213. With permission from Blackwell Publishing, Oxford, UK)

Characteristics	Marine habitat	Freshwater habitat	Groundwater habitat (activity exchanged)
Physical			
Global location	Ocean basins	Continental depressions, valleys, basins	Beneath continental subsoils
Global surface area (%)	70.8	<2	29.0
Global water volume (%)	96.5	0.014	1.7
Residence time for water	~3100 years	2 weeks to ~10 years	2 weeks to 10,000 years
Hydrologic regime	Relatively deep water; very low percentage solids; little, if any, unsaturated zone; hydrologic stratification	Relatively shallow water; low percentage solids; little, if any, unsaturated zone, though some streams are ephemeral; hydrologic stratification	Interstitial water in solid matrix of variable porosity and variable degree of saturation; unsaturated zone may be substantial; hydrologic and geologic stratification
Biological			
Biota	Multicellular and unicellular algae; animals, protists, fungi, and prokaryotes	Multicellular and unicellular algae; animals, protists, fungi, and prokaryotes	Primarily prokaryotes; protists, rare algae, and cave-dwelling animals
Food chain	Photosynthesis, rare chemosynthesis, heterotrophy	Photosynthesis, rare chemosynthesis, heterotrophy	Heterotrophy, chemosynthesis at depth
Nutrient status	Nutrient-poor regions; productivity in upwelling zones	Broad range of oligotrophic, mesotrophic, and eutrophic conditions	Low levels of DOC* and other nutrients common, but many nutrient-rich waters, i.e., beneath landfills
Water flow	Flow paths well defined	Flow paths well defined	Flow paths difficult to define

*DOC, dissolved organic carbon.

Lakes are termed *lentic* environments because their waters are calm and slow. Rivers and streams are *lotic* habitats because they feature water moving in response to gravity. Though only 0.0001% of the water on the Earth occurs in river channels, running waters are of enormous ecological and biogeochemical significance. They play key roles in the hydrologic cycle, deliver

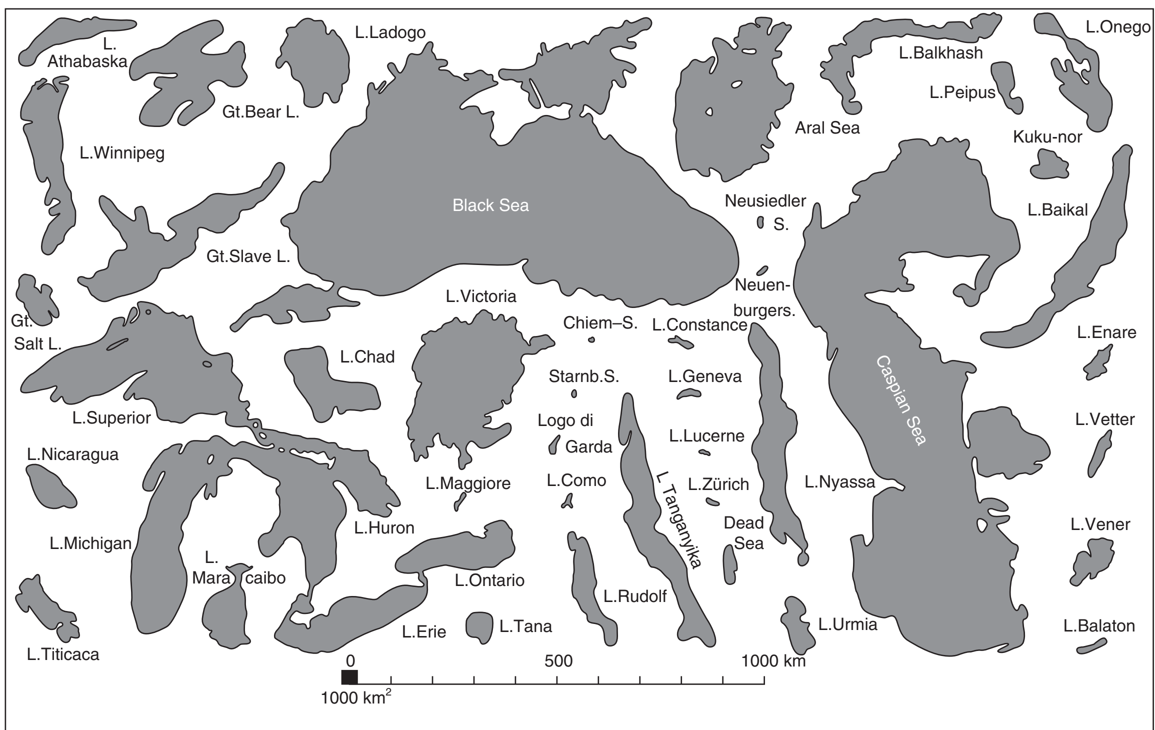


Figure 4.5 Major lakes of the world showing an approximate comparison of the surface areas of many of the larger inland waters, all drawn to the same scale. The Aral Sea has experienced catastrophic reductions in area (more than half of that depicted here) because of diversion of water for agriculture. (Reprinted from Wetzel, R.G. 2001. *Limnology: Lake and River Ecosystems*, 3rd edn. Academic Press, San Diego, CA. Copyright 2001, with permission from Elsevier.)

water across continents, and serve as critical habitats for many important aquatic species. Table 4.4 provides a summary of the catchment sizes and mean annual flows for major rivers in North America, South America, Europe, Africa, and Asia. Immediately derived from atmospheric precipitation, freshwaters generally have low dissolved concentrations of salts. After contacting fallen water, rock and soil generally contribute inorganic constituents to concentrations in the millimolar range (Stumm and Morgan, 1996). The major common ions in freshwaters are SO_4^{2-} (3×10^{-4}M), Cl^- (2.5×10^{-4}M), Ca^{2+} (4×10^{-4}M), Mg^{2+} (3×10^{-4}M), and Na^+ (2.5×10^{-4}M). Obviously, localized conditions throughout the globe contribute other dissolved and suspended aqueous constituents that determine major water quality characteristics such as pH, buffering capacity, dissolved organic matter, particulate organic matter, alkalinity, color, and turbidity.

Ocean waters

Each major oceanic basin (especially the Pacific, Atlantic, Indian, Antarctic, and North Atlantic) offers a unique set of geologic, physical, and biotic

Table 4.4

Catchment size and drainage for selected major river basins of the Earth. (Reprinted from Wetzel, R.G. 2001. *Limnology: Lake and River Ecosystems*, 3rd edn. Academic Press, San Diego, CA. Copyright 2001, with permission from Elsevier)

Rivers by continents	Drainage area (10^3 km^2)	Mean annual flow (m^3/s)
North America	20,700	191,000
Colorado	629	580
Mississippi	3,222	17,300
Rio Grande	352	120
Yukon	932	9,100
South America	17,800	336,000
Amazon	5,578	212,000
Magdalena	241	7,500
Orinoco	881	17,000
Parana	2,305	14,900
San Francisco	673	2,800
Tocantins	907	10,000
Europe	9,800	1,000,000
Danube	817	6,200
Po	70	1,400
Rhine	145	2,200
Rhone	96	1,700
Vistula	197	1,100
Africa	30,300	136,000
Congo	4,015	40,000
Niger	1,114	6,100
Nile	2,980	2,800
Orange	640	350
Senegal	338	700
Zambezi	1,295	7,000
Asia	45,000	435,000
Bramahputra	935	20,000
Ganges	1,060	19,000
Indus	927	5,600
Irrawaddy	430	13,600
Mekong	803	11,000
Oh-Irtysh	2,430	12,000
Tigris-Euphrates	541	1,500
Yangtze	1,943	22,000
Yellow River (Huang Ho)	673	3,300

features. Physical oceanographers have defined key circulatory patterns of ocean waters that have major climatic and biotic implications. Figure 4.6 is a map in plain view of major ocean currents that result from a combination of the Earth's rotation and atmospheric forces. Vertical migration of ocean water also plays a critical role in biosphere function. Figure 4.7 displays a vertical cross-section of ocean circulation patterns centering on Antarctic waters. The

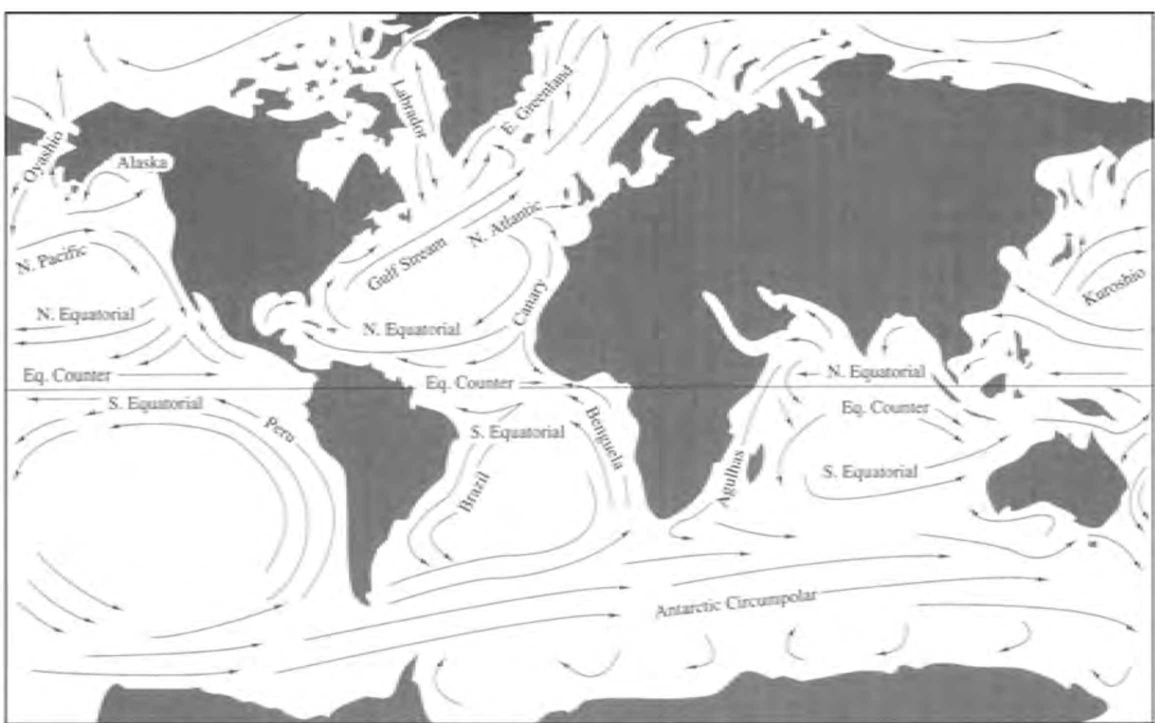


Figure 4.6 Global ocean currents. (Reprinted from Schlesinger, W.H. 1997. *Biogeochemistry: An Analysis of Global Change*, 2nd edn. Academic Press, New York. Copyright 1997, with permission from Elsevier.)

three major circulation loops into the Atlantic, Pacific and Indian Oceans, respectively, are driven by density gradients in which cold, saline waters descend to the ocean bottom. These zones of downwelling are balanced by zones of upwelling, often along continental margins. The horizontal and vertical circulation patterns lead to heterogeneous nutrient distribution patterns (especially for N, P, and Fe) that directly govern the productivity of photosynthetic phytoplankton in ocean waters. Phytoplankton productivity indirectly governs fish productivity and harvest. Figure 4.8 provides an example of ocean productivity – represented by photosynthetic carbon dioxide fixation. Clearly the oceans are nutritionally and biologically heterogeneous.

An accurate perception of the vertical extent of the ocean basins is crucial for understanding these habitats. The vast majority of the oceans are deep, dark, and cold. Ninety percent by volume of ocean water remains at the stable temperature of approximately 3°C. The rest may be influenced by sunlight at the surface, by hydrothermal vents that mark the boundaries of tectonic plates along mid-ocean ridges, and/or by the continental margins. Figure 4.9 displays key categories of zonation at the ocean–continent interface. The *littoral zone* is the extremely shallow periphery influenced by waves and tidal action. The *neritic zone* is a slightly deeper boundary along the continental shelf. Deep ocean waters beyond the continental slope are known as

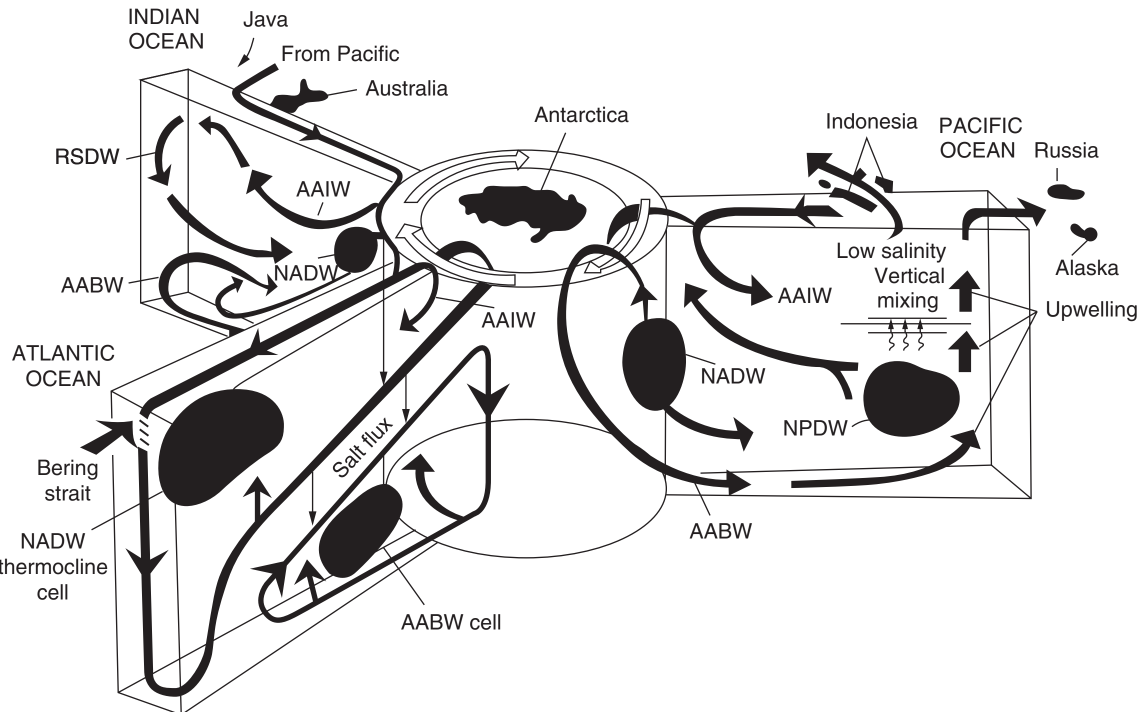


Figure 4.7 Vertical water circulation patterns of the ocean. Thermohaline circulation begins with the sinking cold, salty surface waters in the North Atlantic. The North Atlantic deep water (NADW) thus formed flows toward the south, where it wells up in the Antarctic, cools, mixes with other water types, and sinks again as Antarctic bottom water (AABW). AABW flows north along the ocean floor in all three ocean basins: the Atlantic, Pacific, and Indian. As it flows, its density is gradually reduced by mixing with waters from above. It thus moves upward, to become part of the intermediate-depth, southward-flowing "deep" bodies of water called NADW in the Atlantic, North Pacific deep water (NPDW) in the Pacific, and Red Sea deep water (RSDW) in the Indian Ocean. The combination of AABW flowing to the north and the "deep" water flowing to the south above it forms a deep "conveyor belt" circulation. There is also shallow circulation involving the formation of intermediate-depth water at lower latitudes of the Antarctic (AAIW). This water returns to the Antarctic by a variety of pathways, many not well understood. Also necessary to close the thermohaline circulation is a shallow northward flow to supply the NADW. This probably involves transport around South Africa as well as South America. (From Stumm, J.J. and W. Morgan. 1996. *Aquatic Chemistry*, 3rd edn. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York.)

the *pelagic zone*. Sunlight penetrates the upper pelagic (epipelagic) zone to about 200 m. Beneath this is a zone of very dim light (mesopelagic) that extends an additional 800 m. Deeper still are the bathypelagic and abyssopelagic zones (Figure 4.9). The hadal pelagic extreme is the Mariana Trench (in the Pacific Ocean northeast of Indonesia), 11,000 m below the ocean surface.

Seawater is salty. The salinity is, on average, 35 ppt (parts per thousand) or 35 g of salt per liter – as Na^+ (10.7 g/l), Mg^{2+} (1.29 g/l), Ca^{2+} (0.41 g/l), K^+ (0.4 g/l), Cl^- (19.4 g/l), SO_4^{2-} (2.7 g/l), and HCO_3^- (0.14 g/l), with moderate

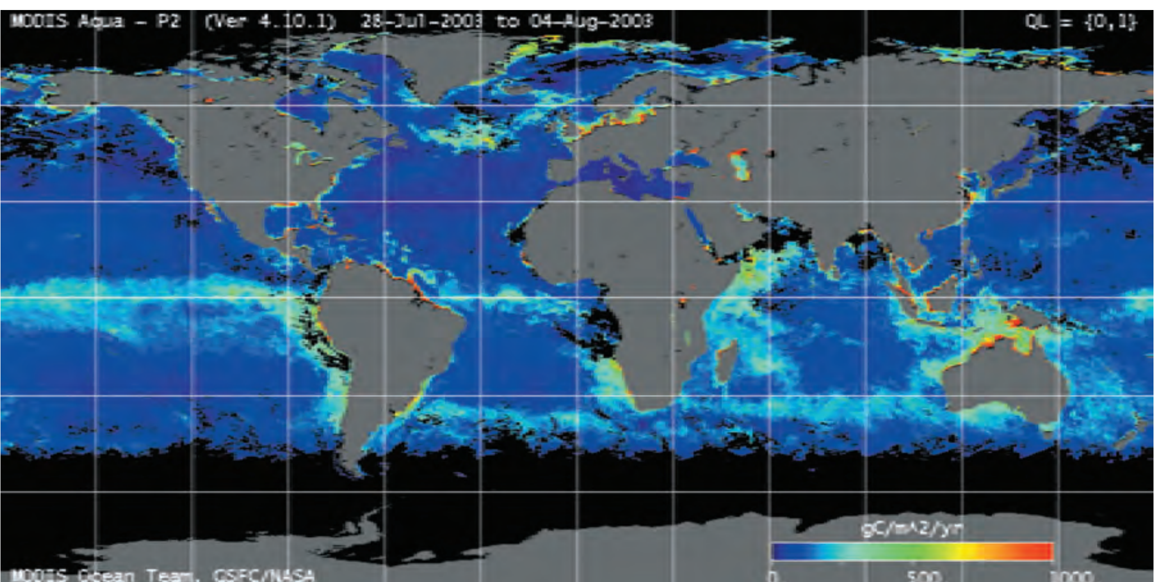


Figure 4.8 World map showing a snapshot of phytoplankton productivity in the oceans, August 2003. (Courtesy of NASA, MODIS Ocean Primary Productivity, with permission.)

contributions from Sr^{2+}, Br^-, and B (Stumm and Morgan, 1996). Although seawater is derived from river waters exiting the continents, the chemistries of the two waters are quite distinctive. Evaporated river water does not resemble the composition of the ocean. Furthermore, the dissolved materials currently present in the oceans are only a small fraction of those that have been delivered to the oceans by rivers over geologic time. Some constituents from incoming river waters are removed from the ocean, as mineral or other precipitates, approximately as fast as they are supplied; this avoids accrual (Stumm and Morgan, 1996). Overall, the composition of seawater is regulated by two key complementary mechanisms: (i) control by chemical equilibria between seawater and oceanic sediments and (ii) kinetic regulation by three interacting rates: supply of individual chemical components, biological processes, and mixing processes.

The global pool of dissolved organic matter (DOM) in ocean water is immense (~700×10^{15} g), roughly equal to the pool or atmospheric CO_2 (Hedges, 2002). A small shift in processes controlling microbial metabolism of DOM to CO_2 and/or the converse (stabilization of DOC so it is not metabolized) has obvious implications for atmospheric chemistry and global climate change (see Section 7.2). Therefore, understanding the chemical nature of marine DOM and its influence on microbial life in the oceans is very important (Kujawinski, 2011). Below is a list of several characteristics of marine DOM that are essential for developing an accurate conceptual view of the oceans as habitats for microorganisms and the processes they catalyze:

(i) The chemical compounds that contribute to the overall pool of DOM are extremely diverse, featuring a broad range of molecular weights

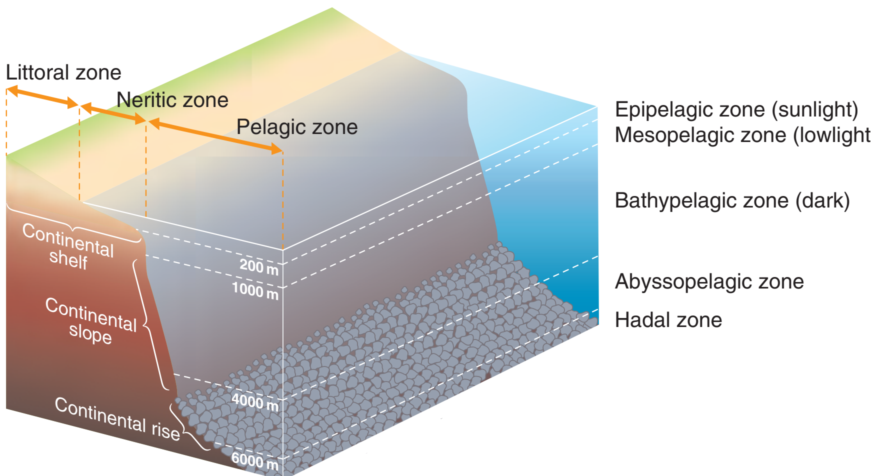


Figure 4.9 Vertical cross-section of the continental shelf.

(low, <1000 Daltons (~70% of total); high, >1000 Daltons (~ 30% of total)) and structures that include organic forms of N, P, and S.

(ii) The few identified low-molecular-weight compounds in DOM include: amino acids, glucose, dimethylsulfoniopropionate, glycine betaine, vanillic acid, ATP, purines, and pyrimidines.

(iii) DOM is produced through a broad diversity of microbial activities that begin with uptake (transport across the cytoplasmic membrane) and continue as biosynthetic and respiratory (energy-generated processes; Kujawinski, 2011).

4.4 SUBSURFACE HABITATS: OCEANIC AND TERRESTRIAL

Subsurface habitats are the deep layers of sediment and rock that extend far beneath soils (on the continents) and the ocean floor. The small pores and harsh conditions that prevail typically support only prokaryotic life, though eukaryotic microorganisms (fungi and protozoa) can occur in the subsurface. Conceptually, subsurface habitats exist as a spherical shell at the biosphere–geosphere interface. The upper edge of subsurface habitats has been defined in various ways: one definition focuses on zones below 8 m on continents and below 10 cm in the oceans (Whitman et al., 1998). The lower boundary of subsurface habitats is a depth of ~4 km, where average temperatures reach ~125°C, which is likely the upper limit for prokaryotic life (Amend and Teske, 2005; Edwards et al., 2012).

A facile way to appreciate the extent and boundaries of subsurface habitats relies upon a plate tectonic map of the globe (Figure 4.10). On the continents, the Earth's crust (granitic in overall composition) is 20–80 km thick with an average age of 3×10^9 years. In contrast, the oceanic crust is basalt, averaging only 10 km in thickness. Due to constant creation of new oceanic crust (at

Figure 4.10 Plate tectonic map of the globe showing major plate boundaries, mid-ocean ridges, and distribution of hydrothermal vent sites. Colored circles show vents with similar animal communities. (Courtesy of E. Paul Oberlander, with permission from Woods Hole Oceanographic Institute.)

spreading centers where fresh lava is emerging) and consumption of oceanic crust (in subduction zones), its average age is 70–100 × 10^6 years. The mid-ocean ridge axis extends globally ~60,000 km. Known locations of deep ocean hydrothermal vents areas are shown in Figure 4.10. The basic unit of hydrothermal activity along the ridge axis is termed a *vent field*. Approximately 280 vent fields have been described to date (this number is sure to grow) and their areal extent is typically 60 × 100 m (Seyfried and Mottl, 1995; German et al., 2004; Schrenk et al., 2010).

Oceanic subsurface

The seafloor features obvious variations in geography (Figure 4.10). Perhaps less obviously, fluid circulation through the seafloor plays a critical role in geologic, geochemical, and microbiological processes (Seyfried and Mottl, 1995; German et al., 2004). Fluid circulation is responsible for large-scale cooling of magma, formation of the oceanic crust, geochemical cycling of the elements, and formation of polymetallic sulfide mineral deposits. Four fluid circulation regions have been identified (Seyfried and Mottl, 1995): (i) the seafloor spreading axis, in which the heat source is magma and sediment cover is generally

lacking; (ii) mid-ocean ridge flanks, in which the crust is still young and hot and sediment cover is thin and patchy; (iii) ocean basins, in which the crust is cooler and sediment cover is thick and continuous; and (iv) subduction zones, where both the seafloor and accumulated sediments are consumed (Lisitzin, 1996; Schrenk et al., 2010). These four somewhat idealized ridge-circulation regions have been further refined by Schrenk et al. (2010), based on rates of magmatic intrusion at the ridges themselves: fast- to intermediate-spreading ridges (East Pacific Rise, Juan de Fuca Ridge, Central Indian Ridge), slow- to ultraslow-spreading ridges (Mid-Atlantic Ridge, Gakkel Ridge), and sediment-covered ridges (Guaymas Basin, Middle Valley, Okinawa Trench). Sediment accumulation directly over a ridge can influence heat flow (insulation may result in higher-than-expected temperatures), may seal off conduits for fluid flow (into and out from the crust), and may also elevate levels of carbon and nutrients utilized by the native microbial communities (Schrenk et al., 2010).

As suggested above, the mature portions of the basaltic ocean floor are overlain by sedimentary deposits. These originate from the overlying water column and/or from the continents. By definition, ocean basins receive water- and wind-borne materials from higher ground. Streams and rivers are major conveyors of dissolved and particulate materials from the continents into the oceans. The settled particulate materials, collectively known as *sediments*, typically have both organic and inorganic components derived from eroding rock and soil. Lisitzin (1996) has mapped and categorized the sediments occurring in the ocean basins. Table 4.5 provides estimates of the thickness, areal extent, and volume of ocean sediments for each of the three major ocean basins. Column 1 of Table 4.5 divides the sediments into seven thickness categories (from <0.1 to >4.0 km). Reading across the table, the data display the areal extent of each thickness category and the corresponding sediment volumes for the Atlantic, Indian, and Pacific basins plus the "world ocean" average. Note that the Atlantic Ocean basin, though less than half the size of the Pacific, has a total sediment volume nearly 50% larger than that of the Pacific. This clearly reflects the relatively large number of high-volume rivers (e.g., Amazon, Mississippi, Congo, Rhine) delivering sediment to the Atlantic Ocean. The figures for average sediment thickness (bottom row of Table 4.5) reinforce the notion that sediments are thin (280 m) in the vast Pacific Ocean basin, relative to the other basins.

Regarding the composition of the ocean sediments, Lisitzin (1996) has created a world map estimating the proportion derived from the continents ("terrigenous") versus that generated within the ocean by indigenous biota (phytoplankton, diatoms, other biomass), termed "autochthronous" (Figure 4.11). The proportion from each source reflects a balance between proximity to major water- or wind-borne particulates and the biotic productivity of the water column. As expected, ocean basins near the mouths of major rivers are highly terrigenous. Surprisingly large areas of the central Pacific Ocean, seemingly out of range for continental impacts, also have been found to be highly terrigenous. Evidently, autochthonous inputs from biota are very low in these locales.

Table 4.5

Thickness, volume, and areal extent of sediments at the ocean floor of three major oceans basins. (From Lisitzin, A.P. 1996. *Oceanic Sedimentation*, p. 20. Copyright 1996, American Geophysical Union. Reprinted by permission of American Geophysical Union)

	Atlantic		Indian		Pacific		World oceans	
Thickness, km	**Area, $km^2 \times 10^6$**	**Volume, $km^3 \times 10^6$**	**Area, $km^2 \times 10^6$**	**Volume, $km^3 \times 10^6$**	**Area, $km^2 \times 10^6$**	**Volume, $km^3 \times 10^6$**	**Area, $km^2 \times 10^6$**	**Volume, $km^3 \times 10^6$**
<0.1	11.46	0.573	18.21	0.910	53.22	2.661	82.89	4.144
01–0.3	7.99	1.198	14.17	2.834	51.80	10.360	73.96	14.392
0.3–0.5	17.47	6.115	11.92	4.768	28.19	11.276	57.58	22.159
0.5–1.0	13.65	10.238	7.58	5.685	27.29	20.468	48.52	36.391
1.0–2.0	13.52	20.208	3.39	5.085	0.14	0.210	17.05	25.575
2.0–4.0	7.83	23.460	1.89	5.670	–	–	9.71	29.130
>4.0	–	–	0.15	1.200	–	–	0.15	1.200
Total	71.92	61.864	57.31	26.152	160.64	44.975	289.86	132.991
		(46.5)*		(19.7)*		(33.8)*		(100)*†
Total oceanic area without seas	82.48	(87.2)†	73.35	(78.1)†	164.8	(97.5)†	325.69	(89)†
Average sediment thickness, m	860		456		280		459	

*Percentage of total volume of runoff.

†Considered or calculated area, percentage of total within each basin; the balance is continental shelf and slope.

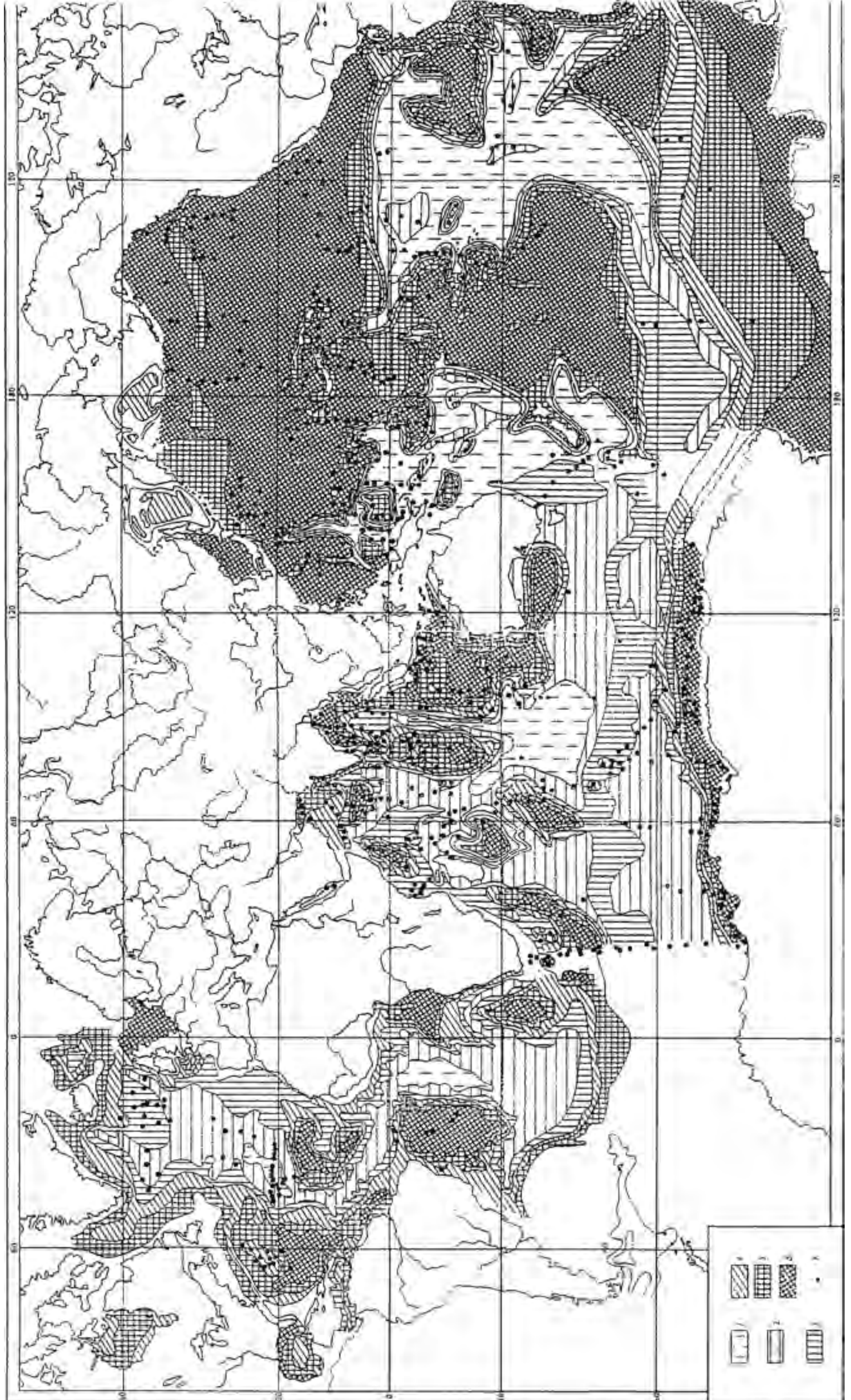


Figure 4.11 Composition of ocean sediments: percent derived from continents (terrigenous) versus autochthonous biotic sources. The six boxes in the key (numbered 1–6) show values from <10% (1, lightest shading) to >90% (6, darkest shading) terrigenous sediment. (From Lisitzin, A.P. 1996. *Oceanic Sedimentation*, pp. 28–29. American Geophysical Union, Washington, DC. Copyright 1996, American Geophysical Union. Reproduced by permission of American Geophysical Union.)

Science and the citizen

The Lost City – Atlantis' myth and reality

Headline news from Plato and Greek mythology

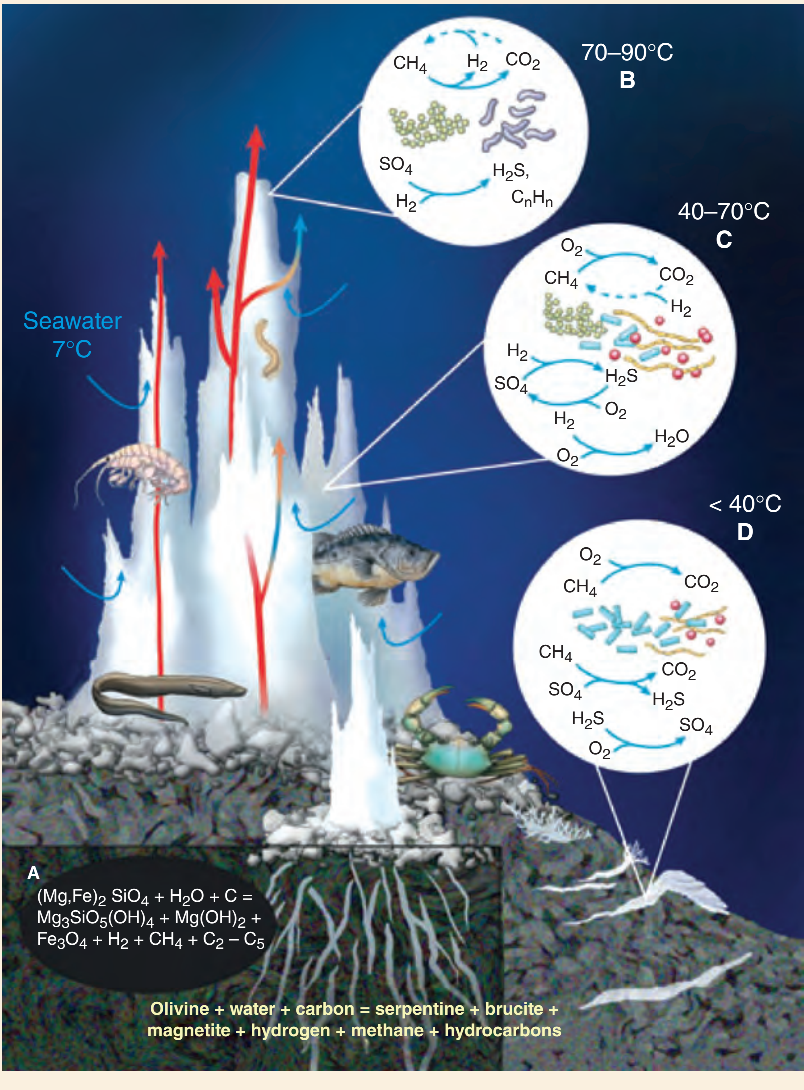
According to the original writings of Plato (Stewart, 1960; Jones, 2002), the mythical civilization of Atlantis was founded by Poseidon (the Greek god of the sea). The center of the civilization was the island of Atlantis, situated in the Atlantic Ocean west of the Straights of Gibraltar (the Pillars of Hercules). The island's mountains surrounded a central plain. By some accounts, the island "was larger than Libya and Asia altogether". The Atlantean royalty, led by Poseidon's son, Atlas (and his descendants), created an extensive Mediterranean dynasty. The royal city included intricate networks of canals, bridges, temples, and palaces. Atlantis' downfall came from three forces: (i) corruption and decadence within Atlantean society; (ii) defeat of the Atlantean army in battles against Athens to the east; and (iii) an earthquake and tidal wave (triggered by judgments of the gods) that caused the island to sink beneath the ocean waves forever.

SCIENCE: A genuine "lost city" beneath the waters of the Atlantic Ocean

Beneath the Atlantic Ocean, midway between North America and North Africa is a plate tectonic boundary known as the mid-Atlantic ridge (MAR) (see Figure 4.10). At latitude 30°N, approximately 15 km to the west of the MAR, is a topographic feature on the seafloor known as the Atlantis massif. On a shelf below the summit of the massif at a depth of ~800 m below sea level is a remarkable geologic formation that was discovered in 2000 (Figure 1).

Figure 1 *(opposite)* A beehive of activity: microbial niches in serpentinization-influenced environments at the Lost City hydrothermal field. (A) Exothermic serpentinization reactions within the subsurface produce fluids of high pH enriched in methane and hydrogen, as well as some hydrocarbons. (B) Environments within the warm interior of carbonate chimneys in contact with end-member hydrothermal fluids host biofilms of *Methanosarcina*-like *Archaea* (green circles). These organisms may play a dominant role in methane production and methane oxidation within the diverse environments present in the chimneys. Bacterial communities within these sites are related to *Firmicutes* (purple rod-like cells). These organisms may be important for sulfate reduction at high temperature and high pH. (C) Moderate-temperature (40–70°C) endolithic environments with areas of sustained mixing of hydrothermal fluids and seawater support a diverse microbial community containing *Methanosarcina*-like *Archaea*, ANME-1 (a methane-oxidizing group of *Archaea*; blue rectangular cells), and bacteria that include ε- and γ-Proteobacteria (yellow filaments and red circles). The oxidation and reduction of sulfur compounds, the consumption and production of methane, and the oxidation of hydrogen most likely dictate the biogeochemistry of these environments. (D) In cooler environments (<40°C) associated with carbonate-filled fractures in serpentinized basement rocks, ANME-1 is the predominant archaeal group. The bacterial populations contain aerobic methanotrophs and sulfur oxidizers (Copyright Taina Litwak 2005, p. 1421 in Boetius, A. 2005. Lost city life. Science **307**:1410–1422, with permission) (Boetius, 2005; Kelley et al., 2005; Schrenk et al., 2010.)

This dramatic "lost city" has resulted from a hydrothermal vent field that is distinctive from the customary "black smoker" hydrothermal vents of the MAR. Beneath this formation, the igneous rock known as peridotite predominates. Unlike the MAR igneous rock (basalt), peridotide is rich in the mineral olivine and is free of the mineral feldspar. Geochemical reactions between hydrothermal fluids and the peridotite form the secondary mineral, serpentine, and give rise to waters that are alkaline and rich in $CaCO_3$, H_2, and CH_4 – with small amounts of hydrocarbons (Figure 1). Spectacular towers, spires, and beehive-like deposits of $CaCO_3$ dominate the seascape. The porous deposits have been colonized by microorganisms (especially *Bacteria* and *Archaea*) that catalyze approximately a dozen biogeochemical reactions. The electron donors for ATP production are H_2, CH_4, H_2S, and hydrocarbons. The electron acceptors are sulfate, CO_2, and O_2.



Research essay assignment

Environmental microbiology can boast of a long history of research events in which the exploration of a new habitat has led to the discovery of unique microbial adaptations. Among these explorations are: the cold, high-pressure deep sea; hydrothermal vents; caves; hot springs; Lake Vostok; the Lost City; and anoxic salty basins beneath the Mediterranean Sea. Search the scientific literature for two such discoveries and prepare an essay comparing them.

Terrestrial subsurface

Continental areas of the Earth are typically composed of materials in the following vertical sequence: the A and B soil horizons; the C soil horizon (from which the other soil horizons may have been derived: Madsen and Ghiorse, 1993; Brady and Weil, 2007); an unsaturated (or vadose) zone (that begins with the C soil horizon and ends at the water table); and a capillary fringe zone residing directly above a saturated zone, which may extend through many different geologic strata (Figure 4.12).

- **Where does the soil habitat end and the subsurface terrestrial habitat begin?**

In some definitions, the subsurface terrestrial habitat begins immediately below the B soil horizon where soil scientists traditionally have felt that photosynthesis-based biological activity ceases (Madsen and Ghiorse, 1993; Brady and Weil, 2007; Chapelle, 2003). It is important to acknowledge, however, that the transition between soil and subsurface terrestrial habitats is not delineated by soil horizons per se. Indeed, plant roots may penetrate the C soil horizon, thereby supplementing the subsurface with photosynthetic carbon compounds that may stimulate microbial activity (Madsen, 1995). Whitman et al. (1998) somewhat arbitrarily chose 8m as the depth of the soil/subsurface boundary.

Beneath the soil, which, by definition, is the zone of *pedogenesis* (soil formation), lie the unsaturated and saturated subsurface zones. This view of the subsurface habitat as being delineated in terms of the degree to which water occupies voids in a porous matrix (if air has been completely displaced by water, the system is "saturated"; if not, the system is "unsaturated") is satisfying, but it is also simplistic, for superimposed upon the degree of water saturation are the geologic, geographic, and climatic characteristics. At a given location on the Earth's surface, the stratigraphy beneath reflects a unique and complex history of geologic, hydrologic, and chemical events (e.g., sedimentation, erosion, volcanism, tectonic activity, dissolution, precipitation, and biogeochemical activity). The result often is a heterogeneous geologic profile whose complexity may be compounded by variations in pore-water chemistry that may stem from localized aberrations in mineral phases or inorganic

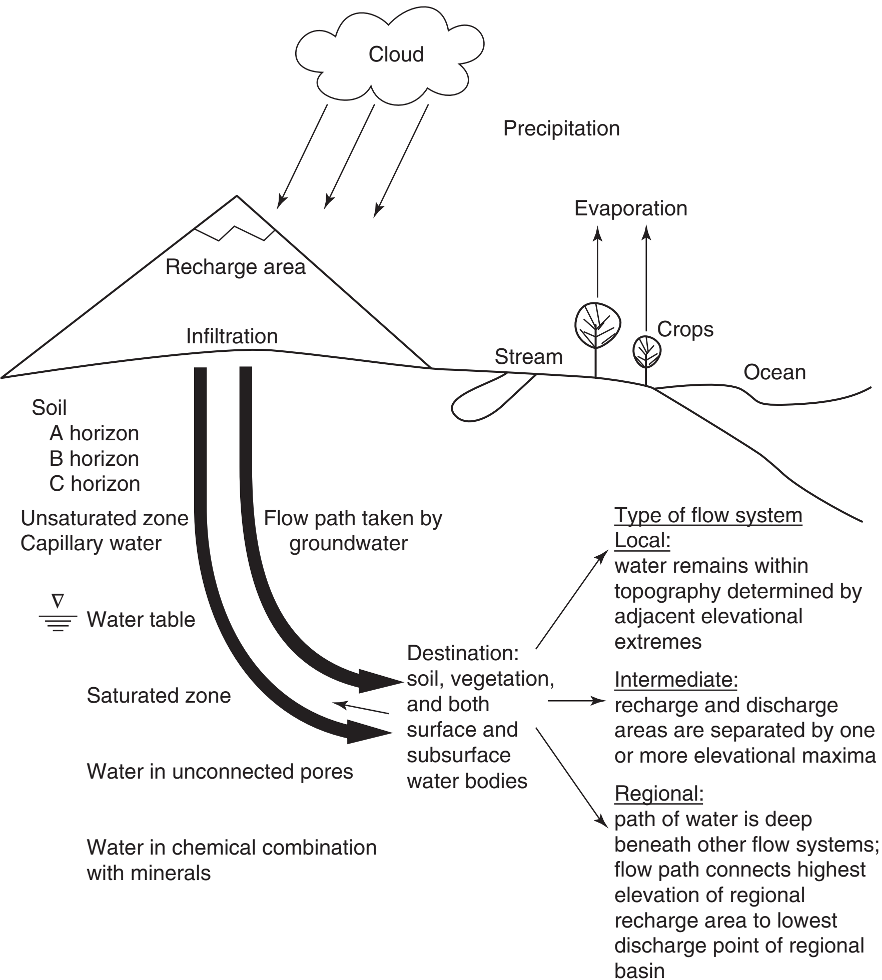


Figure 4.12 Conceptual flow system for understanding the role of soil and subsurface habitats in the hydrologic cycle. (Reprinted from Madsen, E.L. 1995. Impacts of agricultural practices on subsurface microbial ecology. *Adv. Agron*. **54**:1–67. Copyright 1995, with permission from Elsevier.)

or organic solute concentrations. The lower boundary for microbial life in the terrestrial subsurface is likely the depth at which 125°C is reached (~4 km). Out of touch from photosynthetically derived carbon, this "deep biosphere" (Fredrickson and Balkwill, 2006) is dark – reliant upon chemolithoautotrophic metabolism (Edwards et al., 2012). As mentioned in Section 4.3, the large surface area provided by rocks and sediments in the porous matrix may strongly influence the physical and chemical conditions of the groundwater habitat by altering concentrations of dissolved aqueous constituents at the surfaces and by adsorbing microbial cells (van Loosdrecht et al., 1990; Madsen and Ghiorse, 1993; Vaughan and Lloyd, 2011). Adsorption and aqueous equilibrium

reactions are most likely to be influential in the saturated zone. However, many subsurface habitats are dominated by unsaturated zones. In arid climates, the unsaturated zone may be hundreds of meters deep. Rainfall in such desert climates may be insufficient to allow saturated infiltration of soil to reach to the water table, except in restricted low-lying areas (Davis and DeWiest, 1966). Therefore, rather large portions of deserts may have unsaturated zones beneath them with little or no saturated water flux. Under such circumstances, vapor-phase reactions may be the prevalent form of geochemical change. Such conditions have important implications for microbial physiology and activity (Madsen, 1995; Or et al., 2007).

The terrestrial subsurface is an important component of the landscape through which water passes as it cycles among atmosphere, soil, lakes, streams, and oceans (Figure 4.12). Once water has infiltrated below the surface layer of soil, it has several possible fates. It may: (i) return to soil via capillary, gaseous, or saturated transport; (ii) be intercepted by plant roots; (iii) reach streams, lakes, or ponds via saturated flow; (iv) reverse its saturated-flow direction from streams or lakes back into subsurface strata when levels of surface water are high; (v) directly reach the ocean via saturated flow; (vi) become mixed with seawater when groundwater withdrawal in coastal areas causes seawater to intrude inland; or (vii) enter a closed deep continental basin (Figure 4.12; Domenico and Schwartz, 1990; Brooks et al., 2013; Wilderer, 2011). Regardless of the flow path taken through the subsurface, groundwater remains in the biosphere. However, the residence time before water exits the subsurface is highly variable. As mentioned in Section 4.3 and Table 4.3, return of the subsurface water to the soil may occur within a few days or weeks, though return from a deep continental basin may require thousands of years (Freeze and Cherry, 1979; Madsen and Ghiorse, 1993; Wilderer, 2011; Brooks et al., 2013).

In conceptualizing the routes taken by water through the terrestrial segment of the hydrologic cycle, Chapelle's (2003) presentation of local, intermediate, and regional flow system is insightful. Chapelle provides the following definitions for these three flow systems based on relationships among surface topography, large-scale geologic structures, and the depth of water penetration along its path from recharge to discharge areas:

1. A local (shallow flow) system has its recharge area at a topographic high and its discharge area at a topographic low, which are located adjacent to each other.
2. An intermediate system occurs when recharge and discharge areas are separated by one or more topographic highs.
3. In a regional system, deep water flow bypasses local surface topography: the recharge area occupies the regional water divide and the discharge area occurs at the lowest point of the regional basin.

Figure 4.12, which incorporates Chapelle's flow systems, illustrates the spatial and functional relationships between the geologic setting of the subsurface and its most dynamic component, water.

Freeze and Cherry (1979) have presented the idea of "chemical evolution" of groundwater as it passes from the atmosphere in recharge zones along the variety of flow paths such as those depicted in Figure 4.12. As mentioned in Section 4.3, water in the atmosphere begins as pure distillate containing only atmospheric gaseous and atmospheric particulate materials. After contact with soil and deeper subsurface sediments, the chemical composition of the water changes substantially. Not only do components in surface and subsurface matrixes dissolve, volatilize, and precipitate but, as the water reaches zones that are more remote from the atmosphere, complexation and oxidation/reduction reactions also occur. Many of the reactions are strictly geochemical (Albaréde, 2009; Holland and Turekian, 2010; Domenico and Schwartz, 1990; Morel and Hering, 1993; Stumm and Morgan, 1996; Schwarzenbach et al., 2002; Chapelle, 2003), but many are also microbiologically mediated (see, for example, Sections 3.8 and 7.2 to 7.5).

The chemical composition of a given sample of groundwater reflects the integrated history of chemical and microbially mediated biochemical reactions that occur along a given flow path through soil and geologic strata. Because of the diversity of both flow paths and biogeochemical reactions, the composition of groundwater is quite variable. Nonetheless, some generalizations can be made. In aquifers used for drinking-water supplies that are not influenced significantly by human activity, major chemical constituents (>5 mg/l) typically include calcium, magnesium, silica, sodium, bicarbonate, chloride, and sulfate, while minor constituents (0.01–5 mg/l) include iron, potassium, boron, fluoride, and nitrate, with trace amounts (<0.1 mg/l) of many inorganics and organics (including humic acids, fulvic acids, carbohydrates, amino acids, tannins, lignins, hydrocarbons, acetate, and propionate) (Domenico and Schwartz, 1990). However, human activities (including septic systems, landfills, other types of waste disposal, and agricultural practices) may alter the chemistry of groundwater substantially by adding high concentrations of solutes such as both toxic and nontoxic organic carbon compounds and inorganic nutrients. The mineralogy, geochemistry, and dynamics of groundwater habitats set the stage (define the resources) for the types of microbiological processes and interactions presented in Chapter 3.

4.5 DEFINING THE PROKARYOTIC BIOSPHERE: WHERE DO PROKARYOTES OCCUR ON EARTH?

Whitman et al. (1998) completed a global survey of prokaryotic biomass in aquatic habitats, soils, and subsurface sediments, and in the intestinal tracts of selected animals. The approach required the investigators to scrutinize

Table 4.6

Number of prokaryotes in global aquatic habitats. (From Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA* **95**:6578–6583. Copyright 1998, National Academy of Sciences, USA)

Habitat	Volume, cm^3	Cells, ml $\times 10^5$	Total number of cells, $\times 10^{26}$
Marine			
Continental shelf	2.03×10^{20}	5	1.0
Open ocean			
Water, upper 200 m	7.2×10^{22}	5	360
Water, below 200 m	1.3×10^{24}	0.5	650
Sediment, 0–10 cm	3.6×10^{19}	4600	170
Freshwater			
Lakes	1.25×10^{20}	10	1.3
Rivers	1.2×10^{18}	10	0.012
Saline lakes	1.04×10^{20}	10	1.0
Total			1180

reports of the abundances of prokaryotic cells (number per unit volume) in representative habitats and multiply these cell densities by estimated global volumes of each of the habitats. Table 4.6 shows the global survey of prokaryotes in aquatic habitats, exclusive of groundwater. Cell densities in freshwaters (~10^6/ml) average approximately 20 times those of deep ocean water and twice that of the upper ocean and continental-shelf waters. Surface sediment layers of the ocean typically support high numbers of microbial cells (4.6×10^8 cm^3). Despite the low cell density in deep ocean water, this high-volume habitat supports more than half of the global aquatic prokaryotic biomass (Table 4.6). The estimated distribution of prokaryotic biomass in the soils of 12 terrestrial biomes is presented in Table 4.7. Note that the estimated density of soil microorganisms (footnote in Table 4.7) is generally very high (2×10^9 per gram of soil) for most surface soil types, though the density declines significantly with depth. Owing to the large global area and the abundance of bacteria in soil profiles of desert scrub ecosystems, these habitats harbor approximately 25% of the total soil microorganisms. Table 4.8 provides a depth profile and tabulation of prokaryotic biomass in sediments beneath the oceans and continents. Whitman et al. (1998) presumed that 20% of the continental subsurface was unconsolidated sediment – the remaining 80% (rock) was not counted. Clearly the upper 100 m of sediment (Table 4.8, first two rows) contain the majority of subsurface biomass. As will be discussed in Chapter 8 (Sections 8.1 and 8.2), higher life forms are, themselves, habitats available

Table 4.7

Number of prokaryotes in soils of global terrestrial biomes. (From Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA* **95**:6578–6583. Copyright 1998, National Academy of Sciences, USA)

Ecosystem type	Area, $\times 10^{12}$ m^2	Number of cells, $\times 10^{27}$*
Tropical rain forest	17.0	1.0
Tropical seasonal forest	7.5	0.5
Temperate evergreen forest	5.0	0.3
Temperate deciduous forest	7.0	0.4
Boreal forest	12.0	0.6
Woodland and shrubland	8.0	28.1
Savanna	15.0	52.7
Temperate grassland	9.0	31.6
Desert scrub	18.0	63.2
Cultivated land	14.0	49.1
Tundra and alpine	8.0	20.8
Swamps and marsh	2.0	7.3
Total	123.0	255.6

*For forest soils, the number of prokaryotes in the top 1 m was 4×10^7 cells per gram of soil and in the top 1–8 m it was 10^6 cells per gram of soil. For other soils, the number of prokaryotes in the top 1 m was 2×10^9 cells per gram of soil and in the top 1–8 m it was 10^8 cells per gram of soil. The boreal forest, tundra, and alpine soils were only 1 m deep. A cubic meter of soil was taken as 1.3×10^6 g.

Table 4.8

Number of prokaryotes in global unconsolidated subsurface sediments. (From Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA* **95**:6578–6583. Copyright 1998, National Academy of Sciences, USA)

Depth interval,		Number of cells $\times 10^{28}$		
m*	Cells, cm^3 $\times 10^6$	Deep oceans	Continents	Coastal plains
0.1	220.0	66.0	14.5	4.4
10	45.0	121.5	26.6	8.1
100	6.2	18.6	4.1	1.2
200	19.0	57.0	12.5	3.8
300	4.0	12.0	2.6	0.8
400	7.8		10.1	3.2
600	0.95		3.7	1.2
1200	0.61		3.2	1.0
2000	0.44		2.6	0.9
3000	0.34			0.7
Total		275.1	79.9	25.3
Grand total	$380 \times 10^{28} = 3.8 \times 10^{30}$			

*Depth intervals are designated by the upper boundary. Thus, "0.1" represents 0.1–10 m and "3000" represents 3000–4000 m.

Table 4.9

Total number of prokaryotes in some representative animals. (From Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA* **95**:6578–6583. Copyright 1998, National Academy of Sciences, USA)

Animal	Organ	Cells/ml or cells/g	Organ contents*	Number of animals	Number of cells, $\times 10^{23}$
Human	Colon	3.2×10^{11}	220 g	5.6×10^{9}	3.9
Cattle	Rumen	2.1×10^{10}	106 L	1.3×10^{9}	29.0
Sheep and goats	Rumen	4.4×10^{10}	12 L	1.7×10^{9}	9.0
Pigs	Colon	5.4×10^{10}†	9 L	8.8×10^{8}	4.3
	Cecum	2.8×10^{10}	1 L	8.8×10^{8}	0.3
Domestic birds‡	Cecum	9.5×10^{10}	2 g	1.3×10^{10}	0.024
Termites	Hindgut	2.7×10^{6}§		2.4×10^{17}	6.5

*Organ contents in volume or grams of wet weight. For comparison, the volume of the human colon is 0.5 l. For domestic birds, wet weight was calculated from a volume of 2 ml, assuming that 1 ml = 1 g wet weight.
†The direct count was assumed to be 2.7 × viable count.
‡Includes chickens, ducks, and turkeys.
§Per termite.

Table 4.10

Number and biomass of prokaryotes in the world. (From Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA* **95**:6578–6583. Copyright 1998, National Academy of Sciences, USA)

Environment	Number of prokaryotic cells, $\times 10^{28}$	10^{15} g of C in prokaryotes
Aquatic habitats	12	2.2
Oceanic subsurface	355	303
Soil	26	26
Terrestrial subsurface	25–250	22–215
Total	415–640	353–546

for colonization by microorganisms. Table 4.9 shows the tally of prokaryotic biomass that dwells in the digestive tracts of six types of higher organisms – cell densities can be extremely high (3.2×10^{11}/ml in humans).

When Whitman et al. (1998) computed the total global prokaryotic biomass (Table 4.10) the results were astounding: oceanic subsurface prokaryotes constitute well over half of the estimated global total ($353–546 \times 10^{15}$ g of C). The prokaryotic carbon pool is approximately

60–100% of the total carbon found globally in plants. Because prokaryotic biomass is relatively rich in nitrogen and phosphorus, the mass of each of these two essential nutrients in global prokaryotic biomass exceeds that in plants by an order of magnitude.

Several habitat-specific assessments of the prokaryotic biosphere have appeared since Whitman et al.'s (1998) landmark publication. Recent contributions to facts and principles, especially about subsurface microbial life, include:

(i) Kallmeyer et al. (2012), who recalculated the total microbial biomass in oceanic subsurface floor sediments (lowering estimates 10- to 30-fold).

(ii) D'Hondt et al. (2004), who completed a drilling program in the equatorial Pacific Ocean – measuring cell numbers, geochemistry, and estimating rates of physiological activities.

(iii) Edwards et al. (2012), who outlined recent research on the "deep, dark biosphere".

4.6 LIFE AT THE MICRON SCALE: AN EXCURSION INTO THE MICROHABITAT OF SOIL MICROORGANISMS

- **What is it like to be a cell ~1 μm in size in a world whose diameter is 1.2×10^4 km, whose ocean depths can be 11,000 m, and whose soil aggregates are typically ~1 cm?**

We have learned in Section 4.5 that the density of microorganisms in many soils is ~10^9 per gram. Is this a "crowded" state or a "lonely" one? One way to answer this question is to focus on the microbial habitat at the microscale. In this section, we examine the soil habitat because many insights gained from soil are equally applicable to the microbial ecology of sediments and waters.

Appreciating habitat complexity in soil

Scholarly inquiry into the intrinsic properties of soil (pedology), separate from their impacts on plant growth (edaphology), developed significantly in Europe, Russia, and the United States in the nineteenth century (Brady and Weil, 2007). At least two complementary approaches to soil science have progressed simultaneously since then: field approaches to natural history and soil genesis (Buol et al., 2011) and laboratory approaches (chemical, biological, mineralogical, and physical determinations) applied to *soil samples*. Despite advancements in both approaches throughout the twentieth century, McBride (1994) has written, "much of soil science is empirical rather than theoretical in practice. This fact is a result of the extreme complexity and heterogeneity of soils, which are impossible to fully describe or quantify by simple chemical or physical models."

Soils are natural bodies, whose lateral and vertical boundaries usually occur as gradients between mixtures of materials of atmospheric, geologic, aquatic, and/or biotic origin. Soils are open systems subject to fluxes in energy (e.g., sunlight, wind) and materials (e.g., aqueous precipitation, erosion, deposition, inputs of organic compounds from activities of plants, human beings, and other animals). Furthermore, the intrinsic complexity of soil stems from its nature as an assemblage of solid, liquid, gaseous, organic, inorganic, and biological constituents whose chemical composition and random three-dimensional structure have not been completely characterized. In addition to physical complexity, the microbial (*Bacteria*, *Archaea*, fungi, algae, protozoa, viruses; see Chapter 5) physiological processes in soil and their multitude of interactions are dauntingly complicated. Compounding the challenge of understanding soil processes is the fact that abiotic reactions (e.g., precipitation, dilution, hydrolysis) must also be considered when attempting to understand soil biogeochemistry. In a field setting, plants, animals, and microorganisms effect geochemical change.

Attention must also be paid to the fact that the soil properties described above are subject to dynamic changes in time and space. No field setting is homogeneous or static. Regarding spatial inhomogeneity, the physical, chemical, nutritional, and ecological conditions for soil biota undoubtedly vary from the scale of micrometers to kilometers. Regarding temporal variability, in situ processes that directly and indirectly influence fluxes of material into, out of, and within soil are dynamic. Climate-related influences (such as temperature, sunlight, water content, evaporation, and precipitation) are probably major variables that cause temporal variations in biogeochemical processes in soil (Madsen, 1996).

A thermodynamic overview of inorganic soil reactions

Lindsay (1979) provided a unifying thermodynamic overview of soil in which dissolved substances in soil solution are in constant dynamic equilibria with six independent chemical influences: (i) solid mineral phases; (ii) exchangeable ions and surface adsorption; (iii) nutrient uptake by plants; (iv) soil air; (v) organic matter and microorganisms; and (vi) water flux. The mineral phases of soil (typically 90% of the solid matter) have been described as "rock on its way to the ocean" (Lindsay, 1979). Primary minerals (the parent material from which soils are derived) were often formed under conditions of high pressure and temperature. At the Earth's surface, subject to oxidative and hydrolytic weathering, the primary minerals become secondary minerals as ionic species in solution are leached away and the remaining mineral structures seek lower free energy levels in their atomic arrangements. Soils contain numerous minerals, some of which are crystalline, while others are amorphous or metastable. These minerals both respond to and control the dynamic pool of dissolved constituents in soil solution. A detailed discussion of soil mineralogy and equilibria is beyond the scope of this chapter (for this see Bleam 2012;

Lindsay, 1979; Dixon and Weed, 1989; Sposito, 1989; McBride, 1994), but it is critical to appreciate that soil and sediment habitats are in constant chemical transition, albeit at rates that are slow in human terms. Many of the mineral components are thermodynamically unstable and this instability is compounded by additional reaction pathways imposed by (micro) biological processes – especially those driven by plant-derived carbonaceous materials added via photosynthesis.

On size and microscale characterization of microbes in soil

Ladd et al. (1996) and Holden (2011) have reviewed relationships between soil components and the biological activity occurring therein. Both of these authors emphasized the vastness in the range of scale of soil constituents (nine orders of magnitude, from atoms to rocks) and the hierarchical features of soil aggregates that form the three-dimensional fabric of soil. As shown in Figure 4.13, six size-based categories of aggregation were described (Ladd et al., 1996): (i) amorphous minerals develop at the nanometer to angstrom scale; (ii) clay microstructure colloids form at 10^{-7} m diameter; (iii) quasicrystals, domains, and assemblages (10^{-7}–10^{-5} m diameter) form between clay, silt, and smaller particles; (iv) microaggregates (0.1–250 μm diameter) occur between sand, silt, and smaller particles; (v) macroaggregates (250 μm to 25 mm diameter) occur between gravel, sand, and smaller particles; and (vi) clods (>25 mm diameter) occur between rocks, gravel, and smaller particles.

It is the aggregation, the *aggregate behavior*, of soil that contributes to its complexity. Tisdall and Oades (1982) insightfully presented a schematic model of the aggregate organization of soil (Figure 4.14). Emphasized in Figure 4.14 are both the hierarchical scales of soil aggregates and the mechanistically crucial binding agents responsible for aggregate formation. It is clear from Figure 4.14 that soil components of biological origin (typically referred to as soil organic matter – microorganisms plus humic substances; see Section 7.3) play a major role in creating soil structure, and hence the habitat of soil microorganisms. Roots, fungal hyphae, plant debris, fungal debris, bacteria, and humic materials are specifically mentioned in Figure 4.14 because of their structural and nutritional contributions to the soil matrix.

Documentation of soil micromorphology (or "soil fabric"; Ringrose-Voase and Humphreys, 1994) has played a major role in establishing and reinforcing the type of model of soil aggregate organization shown in Figure 4.14. Microscopic procedures that have been applied, whenever possible, to intact soil samples (Foster, 1993; Ladd et al., 1996; Nunan et al., 2002, 2003, 2006; Gregory et al., 2003; Thieme et al., 2003; Young and Crawford, 2004; Taina et al., 2008; Herrmann et al.,; Tippkötter et al., 2009; Rennert et al., 2012; Lehmann et al., 2005) include: epifluorescent analysis of soil thin sections, transmission electron microscopy (TEM), scanning electron microscopy (SEM), electron microprobe analysis (EMP), environmental SEM, nanoscale

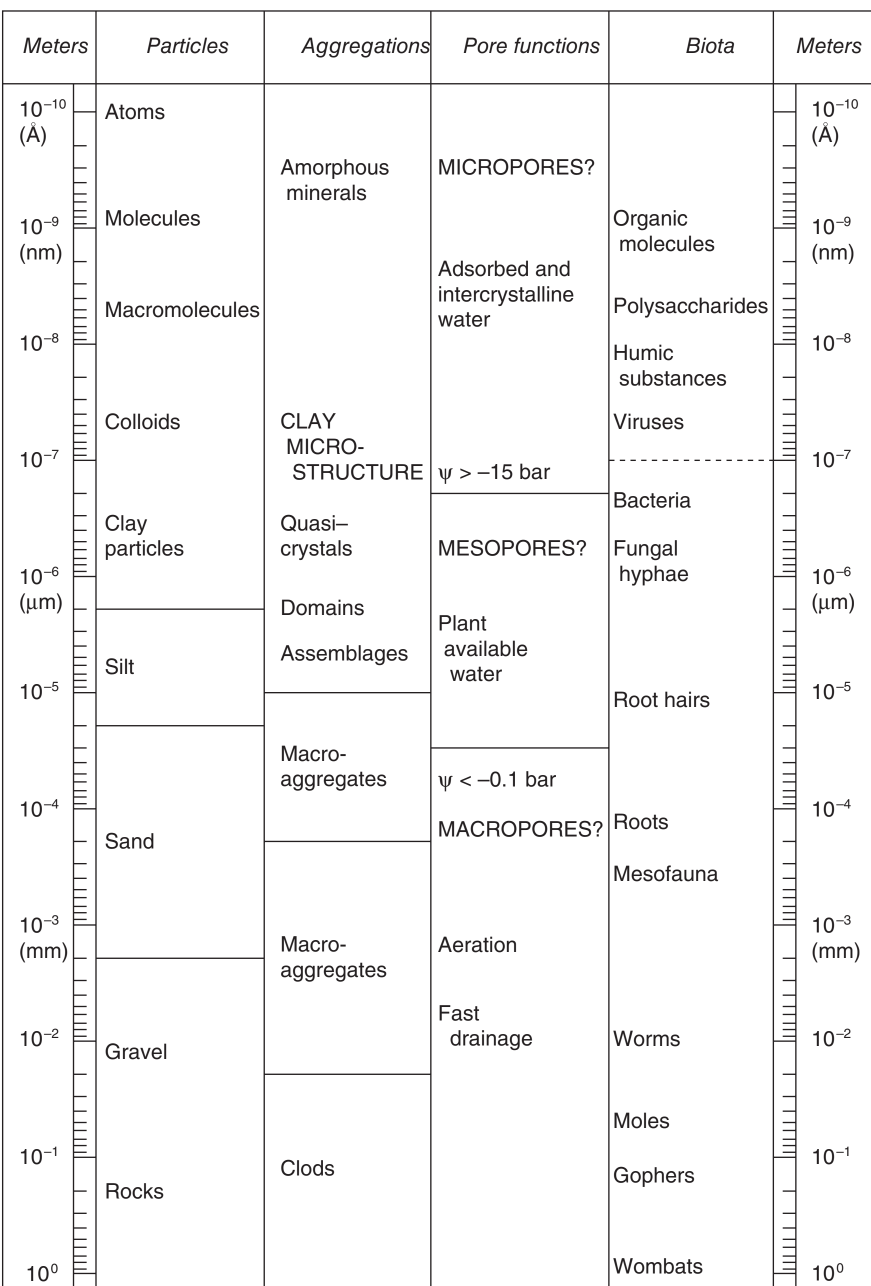


Figure 4.13 The vast range in scale in soil structure and the habitat of soil microorganisms. (From Ladd, J.N., R.C. Foster, P. Nannipieri, and J.M. Oades. 1996. Soil structure and biological activity. In: G. Stotzky and J.-M. Bollag (eds), *Soil Biochemistry*, Vol. 9, pp. 23–78. Copyright 1996, reproduced by permission of Taylor and Francis Group, a division of Informa plc.)

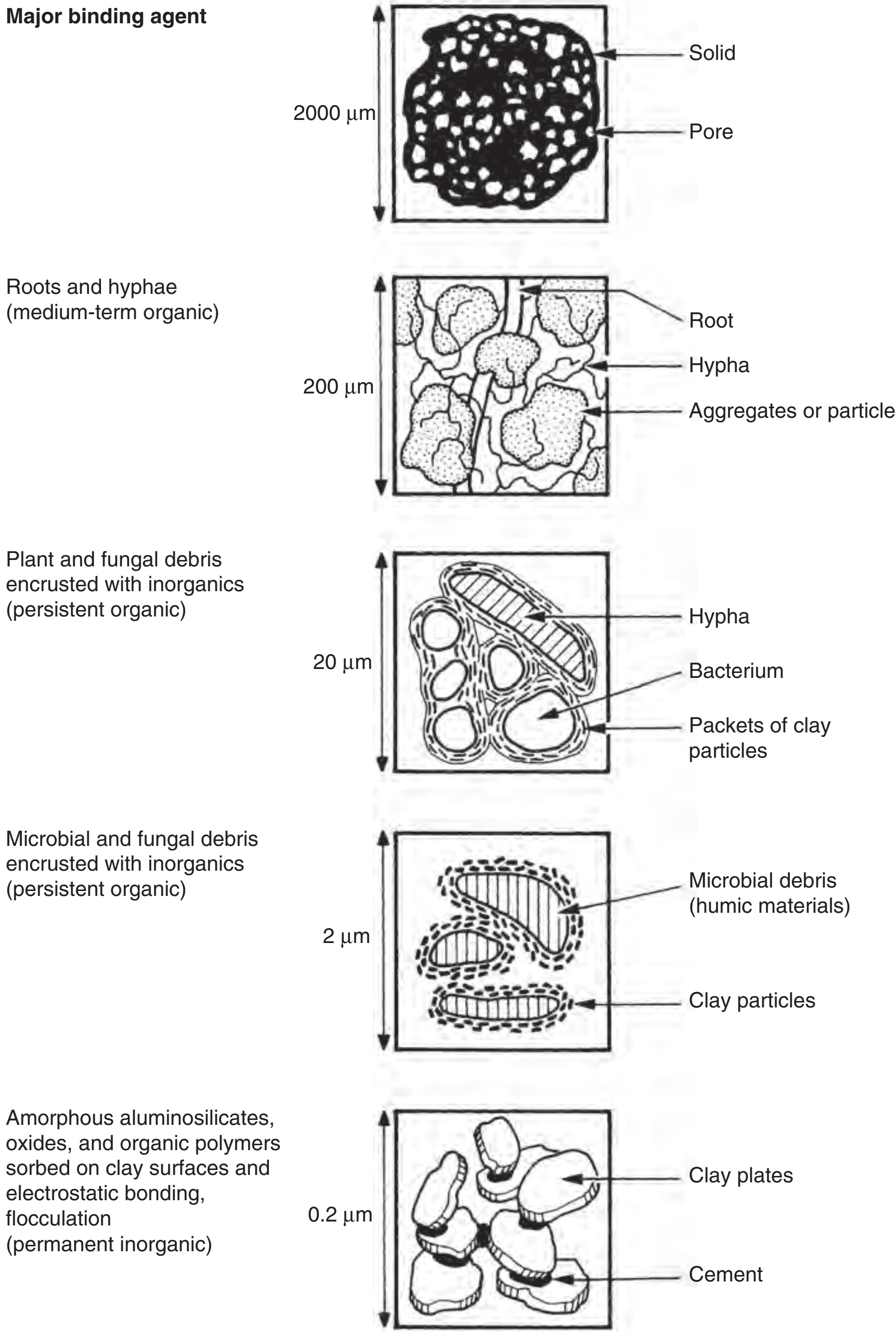


Figure 4.14 Models, at five different scales, of soil components and their contribution to soil structure. (From Tisdall, J.M. and J.M. Oades. 1982. Organic matter and water-stable aggregates in soil. *J. Soil Sci.* **33**:141–163. With permission from Blackwell Publishing, Oxford, UK.)

secondary ion mass spectrometry (nanoSIMS), microfocus X-ray tube computerized tomography, near-edge X-ray absorption fine-structure spectroscopy, and X-ray tomography. These approaches have provided direct observations of intimate associations in soil aggregates of solid surfaces, root hairs, fungi, bacteria, extracellular polysaccharides, clay films (cutans), humic substances, and cellular debris. Such "ultrastructural" studies have revealed that soil microorganisms may occupy only 10^{-3} to 10^{-6} % of the soil surface area; this is because the clay fraction of soils can feature specific surface areas of up to ~10^3 m^2/g. Microorganisms, though present in large numbers (~10^9 cells/g) are neither uniformly nor randomly distributed but, as revealed by TEM of soil sections, have been found clumped near or within cellular residues or in micropores (Ladd et al., 1996). Recent geostatistical analyses of bacteria captured in thin sections of undisturbed soils confirm a patchy, mosaic-like distribution of microorganisms in soil pores (Nunan et al., 2003, 2006; Thieme et al., 2003; Rennert et al., 2012). Although such microscale imaging efforts are revealing, one well-recognized limitation of high-resolution soil microscopy is that each image surveys such a small soil volume that accruing information that is truly representative of bulk soil remains a challenge (Foster, 1993). Fortunately, three-dimensional tomography has recently begun to be used to assemble multiple cross-sectional images (Figure 4.15),

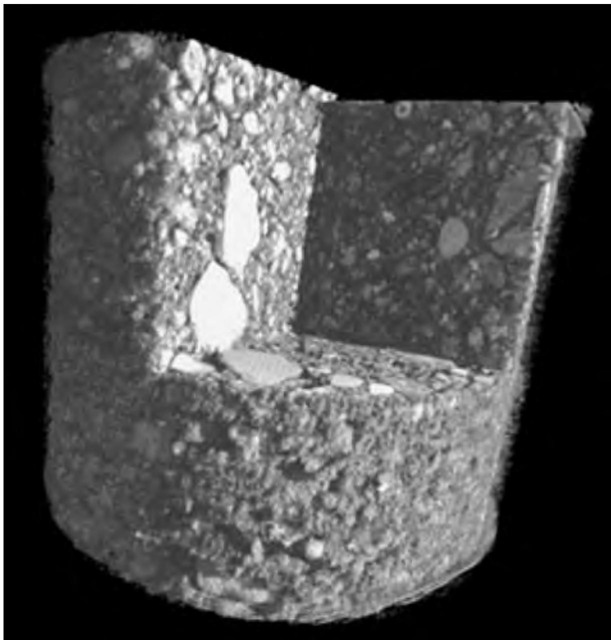


Figure 4.15 A three-dimensional visualization of the soil matrix. (From Young, I.M. and J.W. Crawford. 2004. Interactions and self-organization in the soil–microbe complex. *Science* **304**:1634–1637. Reprinted with permission from AAAS.)

potentially achieving an integrated picture of the micromorphology of the soil habitat.

Holden (2011) has recently summarized state-of-the-art knowledge and investigative methodologies for understanding what regulates microscale bacterial processes in soil. Three fundamental issues that have framed current inquiries and are likely to direct future inquiries are as follows:

(i) Water tension, as a major factor that determines nutrient availability, desiccation stress for cells, and spatial isolation of "segregated microbial patches imposed by restrictive water films".
(ii) Extracellular polymeric substances (EPSs) that are produced by soil microorganisms (during times of nutrient availability) and influence the physical structure of soil by binding to clay and/or to soil organic matter. EPSs can constitute extracellular barriers that protect cells from toxins, predation, starvation, and dehydration, while facilitating adhesion, nutrient and water retention, and community interactions.
(iii) Physical diffusion within the soil matrix, which influences the physiological state of microorganisms and their metabolic activities by governing availability and fluxes of nutrients, dissolved organic carbon, cell–cell communication molecules, and exoenzymes, both within and between both soil pores and water films.

4.7 EXTREME HABITATS FOR LIFE AND MICROBIOLOGICAL ADAPTATIONS

"Extreme" habitats are ones that, from a human point of view, seem inhospitable for life. For humans it may be inconceivable that life may survive and proliferate in hot springs, acid mine drainage, or at the bottom of glaciers. Yet prokaryotes often do (see Section 3.5). These prokaryotic "extremophiles" can be endemic (restricted) to extreme habitats and feature unusual biochemical adaptations not exhibited by prokaryotes dwelling in less extreme habitats. Conditions that prevail in extreme habitats, warranting the extremophile label, include broad ranges in temperature (from approximately −40 to >130°C), pH (from approximately −3.6 to 13), salinity (from rainwater to 5 M NaCl or $MgCl_2$), desiccation (from wet to extremely dry), pressure (from ~0.3 atm on Mt. Everest to 1100 atm in the Mariana Trench), and radiation (e.g., UV light, gamma rays). As discussed in Sections 4.1 to 4.5, contemporary global geography offers wide ranges in altitude, latitude, water availability, temperature, and light intensity. When these conditions are integrated over evolutionary time (see Sections 1.5 and 3.1), it is perhaps predictable that evolution will allow the development of adaptive traits (Cavicchioli, 2002; Cavicchioli and Thomas, 2000; Cavicccchioli et al., 2011; Ng and Cavicchioli, 2011).

Table 4.11 provides a sampling of information derived from microbiological studies of extreme habitats and the traits of microorganisms derived

Table 4.11

Examples of environmentally extreme habitats and extremophiles. (Modified from Cavicchioli, R. 2002. Extremophiles and the search for extraterrestrial life. *Astrobiology* **2**:281–292. With permission from Mary Ann Liebert Publications, Inc.)

Types of extreme condition	Environment	Organism*	Defining growth condition	References
High-temperature growth (hyperthermophile)	Hydrothermal vent	*Pyrodictium*-related strain 121 (A) *Methanopyrus kandleri*	121°C 122°C	Kashefi and Lovley, 2003 Takai et al., 2008
High-temperature growth (hyperthermophile)	Submarine vent, terrestrial, hot spring	*Pyrodictium fumarii* (A)	T_{max} 113°C	Blochl et al., 1997
High-temperature survival	Soil, growth media contaminant	*Moorella thermoacetica* (spore) (B)	2 h, 121°C, 15 psi	Bryer et al., 2000
Cold temperature (psychrophile)	Lake Vostok beneath Antarctic ice sheet	Psychrophilic microbial community	−40°C	Price and Sowers, 2004
Cold temperature (psychrophile)	Snow, lakewater, sediment, ice	Numerous, e.g., Vibrio, Arthrobacter, Pseudomonas (B), and Methanogenium (A) spp. *Planococcus*	−17°C to −15°C	Carpenter et al., 2000; Cavicchioli and Thomas, 2000; Mykytczuk et al., 2013
Cold temperature (psychrophile), high salt, H_2, organic C, ammonia	Lake Vida (Antarctica)	A broad phylogeny of diverse but dormant *Bacteria*; *Psychrobacter* and *Marinobacter* were dominant	−13°C, pH 6.2, salinity 200	Murray et al., 2012
High acid (acidophile)	Acid mine drainage (Iron Mountain, CA)	*Ferroplasma acidarmanis*	pH 0.5	Edwards et al., 2000
High acid (acidophile)	Dry, sulfur-rich, acid soil (solfatara)	*Picrophilus oshimae/torridus* (A)	pH_{opt} 0.7 (1.2 M H_2SO_4)	Schleper et al., 1995; Johnson, 1998
High acid (acidophile) high metal	Acid mine drainage (Rio Tinto, Spain)	Diverse community; *Acidithiobacillus*, *Syntrophobacter* carrying out iron oxidation and sulfate reduction	pH 2.3; high concentrations of Fe, SO_4^{2-}, Cu, Zn	Sánchez-Andrea et al., 2012; Amils et al., 2007
High salt (halophile)	Saline lakes, evaporation ponds, salted foods	Mainly archael halophiles, e.g., Halobacterium and Halorubrum spp.	Saturated salt (up to 5.2 M)	Grant et al., 1998

High salt	Hypersaline basin in eastern Mediterranean Sea	Physiologically active, anaerobic microbial community	5 M $MgCl_2$	van der Wielen et al., 2005; Ferrer et al,.2012
High alkaline (alkaliphile)	Soda lakes (hypersaline lakes rich in Na^+, low in Ca^{2+} and Mg^{2+})	Bacillus spp., *Clostridium paradoxum* (B), and Halorubrum spp. (A)	pH_{opt} >10	Jones et al., 1998
Radiation (radiation tolerant)	Soil, nuclear reactor water core, submarine vent	*Deinococcus radiodurans*, Rubrobacter spp., Kineococcus sp. (B), and *Pyrococcus furiosus* (A)	High γ-, UV, and X-ray radiation (e.g. >5000 Gy γ-radiation and >400 J/m^2 UV)	DiRuggerio et al., 1997; Ferreira et al., 1999; Battista, 2000
Toxicity (toxitolerant)	Toxic waste sites, industrial sites; organic solution and heavy metals	Numerous, e.g., Rhodococcus sp. (B)	Substance-specific (e.g., benzene saturated water)	Isken and de Bont, 1996
High pressure (barophile or piezophiles)	Deep sea	Various, e.g., Photobacterium sp. (B) and Pyrococcus sp. (A)	Deep open ocean or submarine vent (e.g., pressure in Mariana Trench is >1000 atm)	Horikoshi, 1998
Low nutrients (oligotroph)	Pelagic and deep ocean, alpine and Antarctic lakes, various soils	*Sphingopyxis alaskensis* and Caulobacter spp. (B)	Growth with low concentration of nutrients (e.g., <1 mg/L dissolved organic carbon) and inhibited by high concentrations	Schut et al., 1997
Low water activity (xerophile)	Rock surfaces (poikilohydrous), hypersaline, organic fluids (e.g., oils)	Particularly fungi (e.g., *Xeromyces bisporus*) and Archaea (e.g., Halobacterium sp.)	Water activity (a_w) <0.96 (e.g., *X. bisporus* 0.6 and *Halobacterium* 0.75)	Atlas and Bartha, 1998
Rock-dwelling (endolith)	Upper subsurface to deep subterranean	Various, e.g., Methanobacterium subterranean (A) and Pseudomonas sp. (B)	Resident in rock	Atlas and Bartha, 1998

*A, *Archaea*; B, *Bacteria*; Gy, Gray, a unit of ionizing radiation (1 Gy = 100 rad).

therefrom. Thermophilic (high-temperature loving) microorganisms have been discovered in terrestrial hot springs and in submarine hydrothermal vents where hydrostatic pressure allows superheating of the water. Kashefi and Lovley (2003) isolated an organism, strain 121 (related to *Pyrodictium*), capable of growth at 121°C and Takai et al. (2008) showed growth at 122°C by *Methanopyrus kandleri*! This combination of pressure and temperature is that of an autoclave. Regarding cold-tolerant microorganisms, snow- and ice-covered habitats have yielded cultures able to grow at temperatures as low as −17°C. Furthermore, Price and Sowers (2004) have used metabolism–temperature relationships (see Section 3.5) to predict prokaryotic metabolic activity at temperatures as low as −40°C. Lake Vida (Antarctica) is a cold (~13°C) habitat featuring high concentrations of both electron donors (H_2 gas, ammonia, dissolved organic carbon) and acceptors (nitrate, sulfate) along with high metals and salts (Table 4.11); microbial dormancy prevails here, as geochemical evidence of in situ physiological processes was scant (Murray et al., 2012). Regarding acid adaptation, acid-loving microorganisms have been isolated from acid mine drainage (with pH readings of ~0.5) and solfataras (hot, sulfur-rich, terrestrial volcanic vents with pH readings of ~0). High pH and salty habitats such as saline lakes, evaporation ponds, and hypersaline basins have yielded alkaliphilic and halophilic microorganisms able to grow, respectively, at pH >10 and salt concentrations over 5 M. Regarding radiation resistance, *Deinococcus radiodurans* is able to survive gamma-ray exposure 5000 times the dose that is lethal to humans (Schaechter et al., 2006; Pukall et al., 2011). Other traits of prokaryotes featured in Table 4.11 include abilities to tolerate toxic organic compounds, high pressures, low nutrients, desiccation (low water activity), and conditions that prevail in geologic strata.

Comparative physiological and genetic studies of extremophilic prokaryotes have led to mechanistic explanations of how growth and survival occur under the extreme conditions described in Table 4.11. In general, vital molecular components of the cell (especially membranes, proteins, and nucleic acids) need to be modified to achieve functional stability. Table 4.12 provides a summary of adaptive biochemical mechanisms used by prokaryotes exposed to six types of environmental stress. To maintain metabolic function under extreme cold, cells must resist freezing and maintain flexibility in both their membranes and proteins. Membranes of cold-tolerant and pressure-tolerant microorganisms are enriched in unsaturated fatty acids, which confer flexibility, while cold-tolerant enzymes are enriched in polar (not hydrophobic) amino acids and often feature a relatively high proportion of flexible α-helix-type protein tertiary structure (Lengeler et al., 1999; Madigan et al., 2014; Schaechter et al., 2006; Tamburini et al., 2013; Piette et al, 2011; Ng and Cavicchioli, 2011).

Thermophilic microorganisms feature a variety of key adaptations (Table 4.12). Not only do thermophilic enzymes resist denaturation and

Table 4.12

Biochemical adaptations by microorganisms to extreme environmental stresses. (Compiled from Lengeler et al., 1999; Madigan et al., 2014; Schaechter et al., 2006; Cavicchioli et al., 2011; Piette et al., 2011; Tamburini et al., 2013; Ng and Cavicchioli, 2011)

Environmental extreme	Adaptation
Cold	Enzymes are "cold active": • Greater α-helix, polar amino acids • Lesser β-sheet (rigid), hydrophobic amino acids Membranes have more unsaturated fatty acids (stay in fluid state) Cryoprotectants, antifreeze proteins
Heat	Enzymes are "heat stable": • Key amino acid substitutions improve folding stability • Salt bridges (ionic bonds between charged amino acids) • Hydrophobic cores • High content of chaperonin molecules that maintain protein structure Membranes are rich in saturated fatty acids or lack fatty acids entirely (*Archaea*) Special DNA-stabilizing proteins
pH extremes	H^+ stabilizes membranes of acidophiles Intracellular pH kept moderate by membrane transport systems; acid stability in proteins conferred by abundant acidic residues
High salt	Maintain intracellular solutes (pump inorganic ions into cells or concentrate organic solutes) to prevent water loss Exclude salt (expend energy to pump salts out)
High pressure	Enzymes fold so pressure does not alter substrate-binding sites Membranes have more unsaturated fatty acids Membrane composition changes to increase permeability Pressure-controlled gene expression
Radiation/desiccation	Powerful DNA repair machinery. Many copies of DNA repair genes; multiple copies of chromosomes in novel ring-like structure. Mn(II) scavenges reactive oxygen species to minimize DNA damage

coagulation at high temperatures, some enzymes actually function optimally at elevated temperatures. By comparing characteristics of isofunctional enzymes in thermophiles and nonthermophiles, researchers have attributed thermostability to changes in a few key amino acids whose intramolecular hydrogen bonding and salt bridges stabilize tertiary structure. In addition, chaperonin proteins (that facilitate both folding and assembly of catalytic proteins) can play a large role in ensuring

enzymatic functionality of thermophiles. Stabilization of DNA in thermophiles is thought to be the result of DNA supercoiling and association with both DNA-binding proteins and Mg^{2+}. Thermostability of cytoplasmic membranes is imparted by increased proportions of long-chain saturated fatty acids and (in *Archaea*) isoprene-like molecules linked by ether bonds to glycerol phosphate (Lengeler et al., 1999; Madigan et al., 2014; Schaechter et al., 2006).

To accommodate extracellular extremes in pH, microorganisms have developed mechanisms that generally maintain moderate intracellular pH values – often via intracellular cytoplasmic buffering and membrane transport systems that can either pump out protons or pump in the counter ions, K^+ and Na^+ (Table 4.12). Remarkably, some acidophiles actually require high proton concentrations to maintain membrane stability (van de Vossenburg et al., 1998).

The challenge of existence in a high salt environment is maintaining the turgor pressure essential for cell wall growth and adequate water activity in the cytoplasm to maintain routine metabolic function. Halophilic (salt-loving) bacteria and related physiological types (osmophiles, high sugar and xerophiles, lack of water) counterbalance the osmotic flow of water out of the cytoplasm by increasing the internal solute concentrations. Intracellular materials that retain water without disrupting cell physiology are termed "compatible solutes". These can be inorganic salts (e.g., KCl pumped into the cell from the external habitat), but, more often, compatible solutes are organic molecules common to many cellular components (e.g., proteins, carbohydrates, lipids) that can be hydrolyzed intracellularly to yield sugar and amino-acid building blocks that include ectoine, trehalose, glycerol, sucrose, L-proline, D-mannitol, and glycine betaine.

We learned earlier in this chapter about abyssal ocean depths. Hydrostatic pressure adds 1 atm for each 10 m depth of water; thus, the pressure in the Mariana Trench is 1100 atm. Microorganisms capable of tolerating (barotolerant) and those requiring (barophilic) high pressure have been described (Table 4.12). Adaptations to high pressure include: enzymes whose conformation minimizes pressure-related changes in polypeptide folding, a high proportion of unsaturated fatty acids in membranes (to maintain flexibility), and adjustments in the expression of membrane transport proteins (Tamburini et al., 2013).

The astonishing degree of resistance to gamma-irradiation in *Deinococcus radiodurans* is considered the result of several factors that render the organism virtually immune to the ordinarily lethal impact of multiple breaks in DNA expected under conditions of extreme radiation and desiccation (Table 4.12). Multiple DNA repair enzyme systems (including RecA) are active in *D. radiodurans*. The repair mechanisms require the close proximity of an undamaged DNA template. It is thought that undamaged templates are available for this microbe because in each cell there are 4–10 copies of the genome arranged in a dense, ring-like structure (a torroid).

Furthermore, the cells occur in tightly linked clusters of four cells (tetrads), which are able to exchange DNA (Lengeler et al., 1999; Madigan et al., 2014; Schaechter et al., 2006; Pukall et al., 2011).

STUDY QUESTIONS

1 Based on the categories of biomes shown in Figure 4.1, what biome type do you live in?
  (A) Are there virgin tracts of this biome type set aside as park land near you? How have human land-use changes altered local biogeochemistry?
  (B) How have human land-use changes altered biodiversity and ecology?
  (C) How many additional biome types are within 100 km of your home? Within 1000 km? What are they?

2 Do you know the dominant soil order where you live (see Figure 4.4 and Tables 4.1 and 4.2)? How far must you travel to find another soil order? Briefly explain how each of the five factors mentioned in Section 4.2 influences soil development.

3 Use information in Section 4.3 to answer the following:
  (A) If the single outlet (the Angara River) exiting Lake Baikal flows at 60 km^3/year, what is the turnover time of the water in the lake? (Hint: turnover time is the volume of the reservoir divided by the influx or efflux of water.)
  (B) If the river flux out of Lake Titicaca was also 60 km^3/year, what would the turnover time be?
  (C) Consider the implications of turnover time for water pollution events in each lake system. From a management perspective, what are the pros and cons of living near a large water body?

4 Compare the general compositions of freshwaters and ocean waters given in Section 4.3. Identify the major anions in each.
  (A) In light of information in Chapter 3, which of the major anions is physiologically significant for microorganisms? Why?
  (B) Consider that 0.1 g of plant biomass (CH_2O) per liter may dissolve from aquatic macrophytes and be respired by heterotrophic microorganisms native to both freshwater and ocean-water habitats. The CH_2O is the major electron donor. What percent of the dissolved oxygen (assume the initial concentration to be 9 mg/l) would be consumed? After oxygen consumption, how much of the major anion (from part A) would also be respired? (Please show stoichiometries of the reactions and convert all units to millimoles.)
  (C) Given the answer to part B, would you expect distinctive physiological classes of microorganisms in freshwater versus saltwater habitats? If so, why? If not, why not?
  (D) What is the geochemical impact of the heterotrophic activity in fresh- and saltwater habitats? Name the dominant endproduct of electron flow in each habitat.

5 Use Chapelle's terminology (see Section 4.4) to describe the hydrogeologic regime where you live. Where does your drinking water come from? And where does it go?

6 Regarding Section 4.6, are microorganisms "crowded" or "lonely"? Compare and contrast their proximity to one another for a high clay soil and for your own intestines.

7 In acidic environments, the cellular adenosine triphosphate (ATP, energy) demand for maintaining an acceptable intracellular pH may be considerable. For a single class of autotrophic or heterotrophic microorganisms of your choice (see Table 3.3), can you suggest how acidophilic populations are compensated for this additional ATP expense? Please speculate about the evolutionary trade-offs for life in acidic versus neutral habitats.

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5

Microbial Diversity: Who is Here and How do we Know?

*This book is devoted to microorganisms and ways to understand their origins, evolution, ecology, and roles in governing the biogeochemical status of Earth. Prior chapters have focused on planetary history and conditions (Chapters 1 to 4), and on bold themes in prokaryotic and eukaryotic biology (e.g., Section 2.12). This chapter is designed to deliver to the reader fundamental facts and principles about the diversity of microorganisms that dwell in the biosphere. We begin with operational definitions of cultured versus uncultured microorganisms. To take a census, we need to be able to sample, recognize, and classify different microorganisms. Recognition can only occur when we know what microorganisms are and how to distinguish one from another. We must ask and answer two key questions: "What is a microbial species?" and "How does the species concept apply if the only known microbial trait is a 16S rRNA or 18S rRNA gene sequence from the environment?" Molecular phylogeny is discussed and the small subunit rRNA-based tree of life is used to organize a portrayal of biotic diversity. An overview is presented of the major traits and diversity of microbial life: eukaryotic (algae, fungi, protozoa, and slime molds), prokaryotic (*Bacteria *and* Archaea*), and viral. This chapter closes by addressing issues that govern the distribution of microorganisms across Earth's habitat's – biogeography.*

Chapter 5 Outline

5.1 Defining cultured and uncultured microorganisms
5.2 Approaching a census: an introduction to the environmental microbiological "toolbox"
5.3 Criteria for census taking: recognition of distinctive microorganisms (species)
5.4 Proceeding toward census taking and measures of microbial diversity
5.5 The tree of life: our view of evolution's blueprint for biological diversity
5.6 A sampling of key traits of cultured microorganisms from the domains *Eukarya*, *Bacteria*, and *Archaea*
5.7 Placing the "uncultured majority" on the tree of life: what have nonculture-based investigations revealed?
5.8 Viruses: an overview of biology, ecology, and diversity
5.9 Microbial diversity illustrated by genomics, horizontal gene transfer, and cell size
5.10 Biogeography of microorganisms

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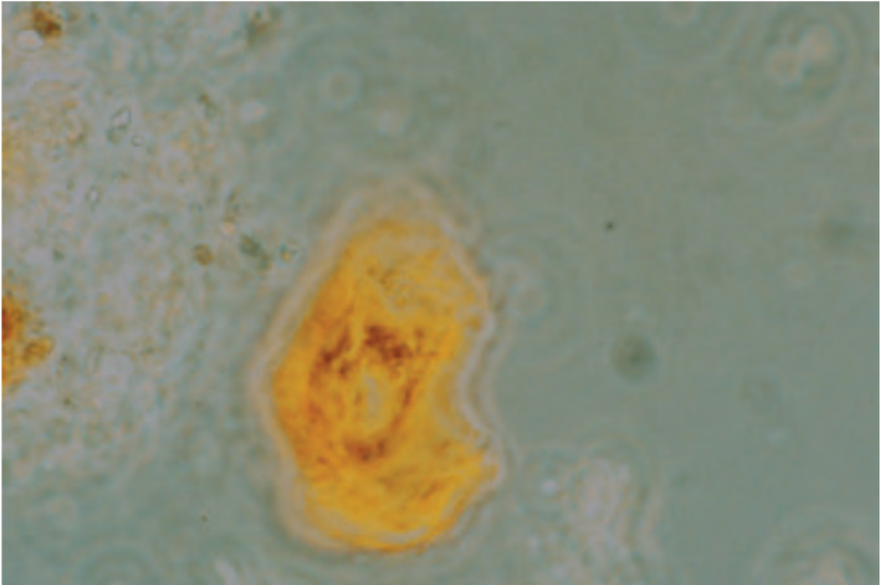
5.1 DEFINING CULTURED AND UNCULTURED MICROORGANISMS

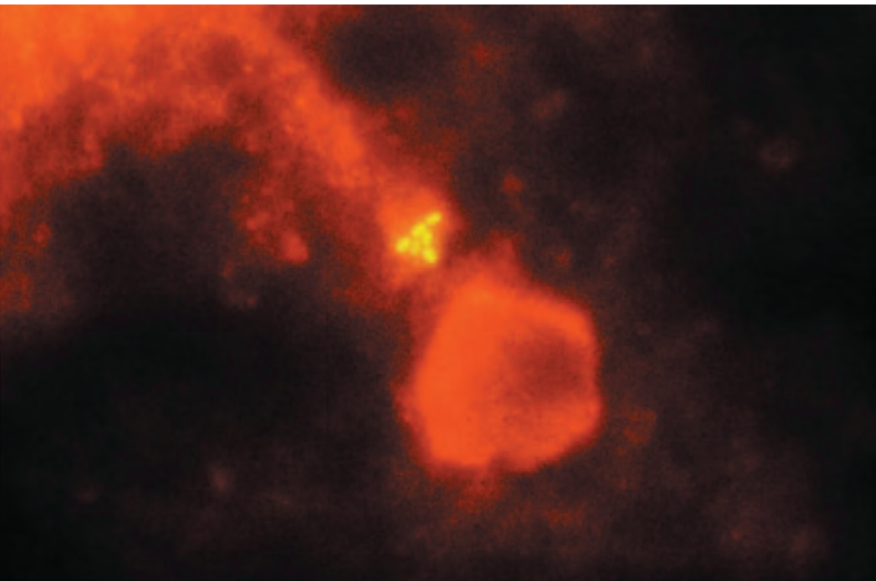
Microscopy provides a foundation for establishing, testing, and honing our understanding of microorganisms in waters, sediments, soils, and other habitats. The first microscopes used by R. Hooke and A. van Leeuwenhoek (see Section 1.3) relied on reflected lamp light for illumination and on both the structure and motility of the microbes to verify their vitality. During the more than three centuries that have elapsed since the pioneering observations of "wee animalcules", the technology has advanced in resolution (e.g., electron microscopy, atomic force microscopy) and in ways to observe and probe the mechanisms of cellular processes (Bonnell, 2001; Murphy, 2001; Braga and Ricci, 2004; Hibbs, 2004; Darby and Hewitson, 2006; Taatjes and Mossman, 2006; Mertz 2010; Morris et al., 2010).

One insightful and widely used approach for visualizing microorganisms in environmental samples is epifluorescent microscopy (Figure 5.1). Figure 5.1a shows an image of soil solids after dilution and spreading on to a glass surface. This type of imaging allows individual particles to be distinguished from one another. However, soils are composed of inorganic components and organic materials (both alive and dead) of widely ranging sizes (see Chapter 4). The vast majority of prokaryotes in soil are not morphologically distinctive – they resemble 1 μm-sized "specks" – as do many inorganic particles and bits of detritus. Fortunately, the unique traits of life (especially the biomarker, DNA) provide an often facile way to distinguish prokaryotes from inert soil particles. Double-stranded DNA has a high affinity for a variety of fluorescent compounds such as acridine orange (AO; N, N, N′, N′-tetramethylacridine-3,6-diamine) and DAPI (4′,6-diaminidino-2-phenylindole). When environmental samples are properly stained with AO or DAPI, individual microorganisms can be imaged and enumerated (Figure 5.1b). Adding 1 g of soil to 99 ml of sterile (cell-free) phosphate buffer disperses the soil particles and the majority of cells become suspended in this 100-fold dilution (Figure 5.2). A 1000-fold dilution of the soil microorganisms can be prepared by transferring 10 ml of the first dilution to 90 ml of sterile buffer. Known (microliter) volumes of such dilutions can be examined using epifluorescent microscopy. Epifluorescent microscopy cell counts in such preparations, adjusted according to the degree of dilution, serve as the basis for microbial abundance data such as those reported in Chapter 4 (Section 4.5, Tables 4.6 to 4.10).

The soil dilutions described above for an epifluorescent microscopy assay can also be used in cultivation-based isolation and enumeration procedures (Figure 5.2). For these, 0.1 ml of a given soil dilution is spread on to the surface of a solid agar medium in a Petri dish. If 10 colonies grow from 0.1 ml of a 1000-fold dilution of 1 g of soil, then we infer a viable plate count of 10^5 microorganisms per gram of soil (each colony is presumed to be derived from a single cell; each of the 10 colonies represented are one ten-thousandth of the microorganisms in the original 1 g of soil). Thus, viable plate counts of microorganisms can be directly compared to



(a)



(b)

Figure 5.1 Microscopic images of a soil sample after dilution and smearing on to a glass slide. (a) Phase-contrast image showing soil solids. (b) Epifluorescence image of the same field after staining with a dye (acridine orange) that specifically binds to nucleic acids. Microorganisms are revealed as bright fluorescent cells. (From W.C. Ghiorse, Cornell University, with permission.)

epifluorescent microscopic counts of microorganisms from identical environmental samples. Nearly inevitably, when this comparison is made, the number of cultured cells on agar plates is 100–1000 times fewer than the total number of microscopic cells (Figure 5.2).

Figure 5.3 presents a scheme that operationally defines six categories of microorganisms in nature based on culturability. The small subset of the total microorganisms that form colonies on agar plates are, by definition, ones that grow. As we learned in Chapter 3, microbial growth requires proper resources, especially a carbon source, nutrients, electron donors, and electron acceptors. As we learned in Chapters 3 and 4, biosphere resources providing selective pressures for microbial growth over evolutionary time are diverse, complex, and poorly characterized. Furthermore, the resources may be metabolized via cooperation among microbial populations. Thus, the first dichotomous branch in the scheme of Figure 5.3 reflects how well microbiologists can devise growth media that match the needs of members of the microbial world.

1 *Cultured microorganisms* are those that have been successfully isolated and purified in the laboratory. These are represented in culture collections such as those in the United States and Germany, Belgium, the Netherlands, Japan, China, the United Kingdom, France, and Poland (e.g., American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)). Information produced from these cultured, model organisms is the basis for ~99% of what is known about microbiology. The textbooks are written about these organisms and their structures,

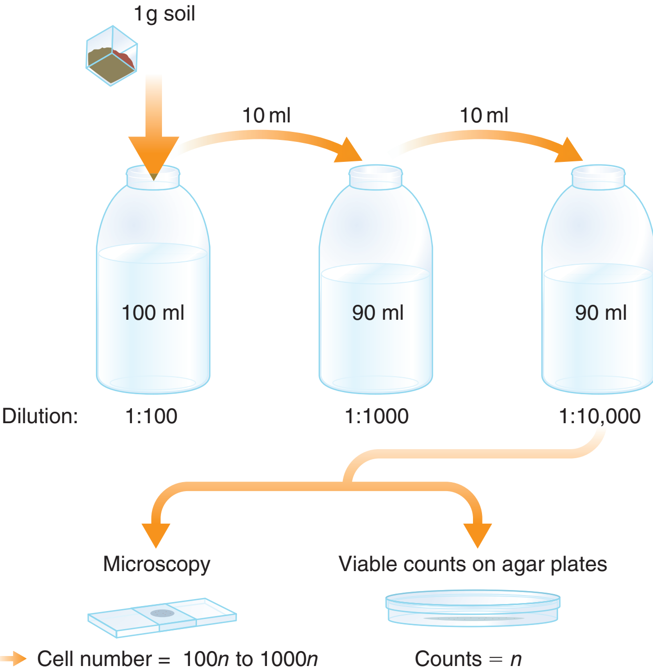


Figure 5.2 Preparation of soil dilutions in sterile buffer. The contrasts between the number of microscopic microorganisms and the number that grow on solid agar media has been termed the "great plate count anomaly".

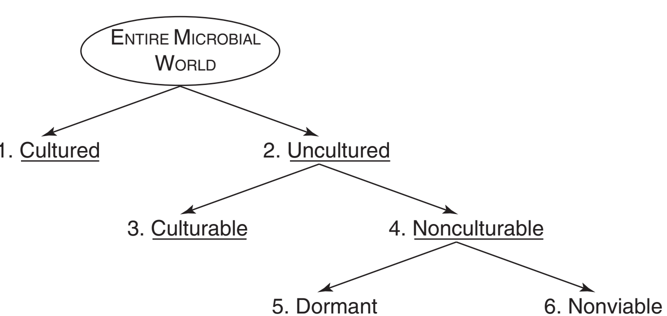


Figure 5.3 Six categories of culturability for microorganisms in nature. The categories are operationally defined – based on techniques of detection that include microscopy and both traditional and novel procedures for growth and isolation. See text for details.

Table 5.1

Tabulation of cultured prokaryotic taxa. (Modified and updated from Garrity, G.M., T.G. Libum, and J.A. Ball. 2005. The revised roadmap to the manual. In: D.J. Brenner, N.R. Krieg, and J.T. Staley (eds), *Bergey's Manual of Systemic Bacteriology*, Vol. 2, Part A, 2nd edn, table 1, p. 163. Springer-Verlag, New York. With kind permission of Springer Science and Business Media)

	Domain		
Taxonomic category	***Bacteria***	***Archaea***	**Total**
Phyla	24 (26[a])	5[b]	28
Class	32	9	41
Subclass	5	0	5
Order	75	13	88
Suborder	17	0	17
Family	217	23	240
Genera	1115 (2,611)[a]	79 (140)[a]	1194 (2751)[a]
Species	6185 (13,327)[a]	281 (524)[a]	6466 (13,851)[a]

[a]National Center for Biotechnology Information (NCBI) 2014 tabulation. http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.htnl.index.cgi.
[b]Number reflects the addition of *Korarchaeota, Thaumarchaeota.,* and *Nanoarchaeota*.

growth characteristics, physiology, pathogenicity, genetics, etc. The tally and diversity of cultured microorganisms is constantly growing because microbiologists are constantly devising new ways to meet the nutritional and physiological needs of microorganisms moved from their native habitats (e.g., soil, sediment, waters, gastrointestinal tracts of animals) to artificial laboratory media. As of 2005, the number of distinctive cultured prokaryotic microorganisms (e.g., species of *Bacteria* and *Archaea*) stood at 6466 – falling into 1194 genera and 240 taxonomic families (Table 5.1; Garrity et al., 2005). By 2012–2014, the total tallies of cultivated species ranged from >10,000 (Amaral-Zettler et al., 2010) to ~14,000 (Table 5.1).

2 *Uncultured microorganisms* are the remainder. No one knows the full extent of microbial diversity in the biosphere. Estimates (see Section 5.4) are that the number of uncultured species (the "uncultured microbial majority"; see Rappé and Giovannoni, 2003; Gans et al., 2005; Pace, 2009; Sogin, 2009; Auguett et al., 2010; Pace et al., 2012; Pawlowska et al., 2012; Rinke et al., 2014) may be 10^4 times that of the cultured minority. Uncultured microorganisms are the ones for which no appropriate growth medium has been devised, but conceptually the uncultured category can be further dissected (see the second dichotomous branch in Figure 5.3).

3 *Culturable microorganisms* are the ones that will become cultured when a clever microbiologist devises a growth medium that matches the organism's nutritional needs. Key physical and chemical growth conditions

must also be provided. *Thus, from a taxonomic point of view there is no such thing as an "unculturable microorganism"*.

4 *Nonculturable microorganisms* are ones present in soils, sediment, waters, or other habitats whose physiological state prevents them from being cultured. The third dichotomous branch of Figure 5.3 splits "nonculturables" into two categories: dormant and nonviable.
5 *Dormant cells* (previously defined in Sections 3.4 and 3.5) may have been quiescent for so long, with such a small proportion of the cellular components intact, that growth is not possible. A poorly understood resuscitation step may be required before dormant cells can be cultured (Hoefman et al., 2012; del MarLleo et al., 2005; Oliver, 2005; Vora et al., 2005).
6 *Nonviable cells* are ones that cannot be resuscitated. They may be visible in a microscope but are irreparably damaged or moribund. These cells are, effectively, dead.

5.2 APPROACHING A CENSUS: AN INTRODUCTION TO THE ENVIRONMENTAL MICROBIOLOGICAL "TOOLBOX"

- **How would you take a census of cookies in a bakery?**
- **How would you document the diversity of the plant species in a 100 m^2 of forest?**

To achieve these two seemingly simple goals, you need at least three basic abilities: (i) a way to sample the cookies or plants; (ii) a way to recognize the cookies or plants – to differentiate them from other things; and (iii) a way to distinguish different classes of cookies or plants from their peers. Once the three abilities are established, you simply count the different classes of distinctive cookies or plants as they are being sampled. Here it is helpful to recognize and define several aspects of census taking:

- The sampling step is unlikely to be 100% effective; thus, we estimate the total based upon the sampled subset.
- One measure of diversity is the *richness*: the total number of different types of taxa (cookies, plants, or microbial species).
- Another measure of diversity is *evenness*: this encompasses both the variety of taxa and their relative abundances.

In Section 4.5 we learned that soil habitats possess ~10^9 microorganisms in a single gram. Furthermore (see Section 5.1), the vast majority of microorganisms have not yet been cultured.

- **Given this situation, is it reasonable to even attempt an assessment of microbial diversity?**
- **Do we microbiologists have anything resembling the three essential abilities (sample, recognize, classify) required to conduct a census of cookies and plants?**

Box 5.1 introduces the four types of fundamental tools (methodological approaches) available to environmental microbiologists: microscopy, cultivation, physiological incubations, and biomarkers. All four of the approaches can be brought to bear on the goal of taking a census of microorganisms present in a liter of lake water, in a gram of soil, and/or in the biosphere at large.

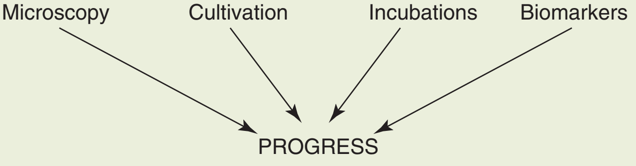
Box 5.1

Introducing the four fundamental methodological approaches in environmental microbiology

There are four fundamental methodological approaches that generate information addressing environmental microbiological questions and issues. These approaches are:

1. *Microscopy*: direct imaging at the microscale to verify the presence of microbial cells. Microscopy provides direct, irrefutable data on the total abundance of microorganisms in environmental samples. Recent technological advancements in the resolution and types of information gathered during imaging aim to identify individual cells and probe their biogeochemical activities.
2. *Cultivation*: environmental matrixes (e.g., soil, sediment, water) are diluted and suspended microorganisms are transferred to liquid or solid media where nutrients are provided – with hopes that the cells present will grow. Assays for assessing growth include colony formation (on agar plates), increased cell numbers in liquid media, and chemical endpoints indicative of a physiological process. By counting individual colonies growing from known dilutions of environmental samples, different types of microorganisms can be enumerated.
3. *Physiological incubations*: microbial populations occur as complex communities in environmental samples. Environmental samples can be brought into the laboratory, sealed in vessels, and subjected to assays that demonstrate the physiological potential of the microorganisms, such as production of CO_2 or CH_4, nitrogen fixation, sulfate reduction, or metabolism of pollutant compounds.
4. *Biomarker extraction and analysis*: prokaryote (or eukaryote)-specific molecular structures (e.g., nucleic acids, proteins, lipids) can be directly extracted from environmental samples. After analysis, these provide insightful clues about the presence and activity of microorganisms (see also Section 2.2).

No single approach leads to a thorough understanding or answer to a given question. Information from all four approaches can complement and confirm one another. When this confluence occurs, the discipline of environmental microbiology is advanced.



1. *Microscopy* (see the discussion above and Box 5.1) allows images of microorganisms to be obtained directly from environmental samples. Such data are essential for enumeration of total cells in environmental samples. Technological innovations in microscopy, such as flow cytometry and other procedures (see Section 6.7 and Table 6.4), have the potential to gather additional information about the types of cells present (such as size and RNA content) and if the cells bind to probe molecules specifically targeting structures such as surface antigens or rRNA.
2. *Cultivation* (see the discussion above and Box 5.1) allows microorganisms to be grown and isolated when their nutritional requirements are met. The growth response of quantitatively diluted microorganisms can reveal the abundances of particular microbiological types.
3. *Physiological incubations* (Box 5.1) document the presence and biogeochemical potential of microorganisms in vessels containing either diluted or undiluted environmental samples. Under controlled conditions, physical (e.g., growth displayed as turbidity) or chemical (e.g., consumption of a carbon source or an electron acceptor; production of CO_2, acidity, or metabolites) changes characteristic of physiological processes can be easily recognized. The incubations display endpoints for a given physiological process (e.g., methanogenesis or sulfate reduction). When environmental samples are quantitatively diluted and used in such incubations, the abundance of a given functional type of microorganism can be quantitatively assessed. (If the endpoint is reached in highly dilute preparations, then the number of active organisms initially present in the environmental sample was high.) All such physiological assays have the same caveat as cultivation-based approaches because if nutritional needs are not met, the physiological potential of microorganisms cannot be expressed.
4. *Biomarkers* have revolutionized our understanding of the microbial world during the last two decades. Biomarkers go hand in hand with microscopy, as the mainstay of *noncultivation-based procedures* for taking a microbiological census in natural habitats such as water, sediment, and soil. We saw in Chapter 2 (Section 2.1, Table 2.2, and Box 2.2) that biomarkers (especially stable isotopic ratios and molecular fossils) have proven essential for discovering clues about the ancient history of prokaryotic life. The biomarkers that have proven most insightful for contemporary microorganisms are nucleic acids – especially the sequences of taxonomically and evolutionarily insightful ribosomal RNA genes (i.e., small subunit 16S rRNA (in prokaryotes) and 18S rDNA (in eukaryotes); see Section 5.5). Nucleic acid biomarkers have had a profound impact on environmental microbiology for three major reasons: (i) fine distinctions between nucleotide sequences can be made when many of these extracted biomarkers are compared; (ii) the sensitivity for detecting (cloning and sequencing) nucleic acid is very high due to the applicability of the polymerase chain reaction (PCR) in recovering sequences derived from environmental samples; and (iii) identical molecular criteria for recognizing and comparing microorganisms apply to sequences derived from both cultivated and noncultivated sources. In addition to

nucleic acids, other biomarkers (to be discussed in Sections 6.7 to 6.11) include membrane lipids, proteins, and metabolites.

At the apex of sought biomarkers is the genome sequence (the entire genetic blueprint of an individual species, or microbial population). The traditional means for obtaining a genome sequence has relied upon successful cultivation of pure cultures of the microorganism of interest. Recently, both single-cell genomics (see Section 6.9) and draft-genome assembly from metagenomic data (see Section 6.10) have bypassed the cultivation step as a prerequisite for acquiring genomic information (Wrighton et al., 2012; Hess et al., 2011; Marcy et al., 2007; Rinke et al., 2013, 2014).

5.3 CRITERIA FOR CENSUS TAKING: RECOGNITION OF DISTINCTIVE MICROORGANISMS (SPECIES)

Cultured microorganisms (defined in Section 5.1) are analogous to domesticated animals – these are the ones we can breed, manage, and study. It follows that uncultured microorganisms are analogous to wild animals – we get fleeting glimpses of them during our forays into their habitats. We know that the fundamentals of domesticated animal reproduction, speciation, and taxonomy apply to wild animals. Microbial ecologists also apply (to the degree possible) the taxonomic rules of domesticated microorganisms to those that are not yet cultured.

The concept of “microbial species” becomes crucial in our efforts to take a census of microorganisms in nature. The criteria for species allows us to recognize and classify, and hence document the presence and abundance of, different types of microorganisms in soils, sediments, waters, and other habitats. For cultured microorganisms, Rosselló-Mora and Amann (2001) have stated that species categories should be based on both phylogenetic and phenotypic traits. Each species is:

> … a genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property.

A perceptive reader will notice that the above definition is imbued with legalistic connotations. The wording of the definition must be defensive and precise because microbial (and other) taxonomists are imposing an artificial system of categories and nomenclature on the continuum of biological variations delivered by evolution. Debates on how best to define microbial species are ongoing (e.g., Ward, 1998; Rosselló-Mora and Amann, 2001; Cohan, 2002; Stackebrandt et al., 2002; Kassen and Rainey, 2004; Gevers et al., 2005; Rainey and Oren, 2011; Fraser et al, 2009; Gribaldo and Brochier, 2009; Richter and Rosselló-Mora, 2009; Yarza et al., 2014; Konstantinidis and Stackebrandt, 2013). Nonetheless, there is

Box 5.2

Criteria that establish if microorganisms belong to the same species

There are three main taxonomic and molecular criteria used for deciding that two cultivated microorganisms belong to the same species (Rosselló-Mora and Amann, 2001; Stackebrandt et al., 2002; Gevers et al., 2005; Richter and Rosselló -Mora, 2009; Yarza et al., 2014):

1. *DNA–DNA hybridization*: (i) 70% or greater DNA–DNA relatedness in whole genome reannealing tests and (ii) <5 °C difference in the temperature of DNA helix dissociation (ΔT_m) for the two strains.
2. *16S rRNA gene*: >98.7% identity in sequence (≤1.3% difference; Yarza et al., 2014). This criterion is not absolute, as many phenotypically distinctive species have been discovered to have >98.7% 16S rRNA gene sequence identity. For genus-, family-, order-, class-, and phylum-level designations, sequence identities of 94.5%, 86.5%, 82.0%, 78.5%, and 75% or lower have been proposed (Yarza et al., 2014).
3. *Whole genome sequence comparison*: average nucleotide identity (ANI) is a genome–genome sequence-comparison parameter that has emerged for defining species (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Mora, 2009). An ANI range of 94–96% for protein-coding genes with shared function defines common species.

As stated in Section 3.2, many strains of the same species can differ substantially in genome size and content. Identifying the critical phenotypic and genotypic traits that define "species" is an ongoing challenge, largely because subjective judgments must be made by taxonomists. A multigene strategy, known as multilocus sequence analysis (MLSA) has immense promise for developing new definitive criteria.

widespread agreement on the genetic criteria that define a species of cultivated bacteria (Box 5.2). Genomic hybridization determines the degree to which DNA strands from two different bacteria hybridize to one another relative to themselves (Stackebrandt et al., 2002). If the percent hybridization (a direct measure of sequence similarity) is 70–100%, the bacteria are deemed to be the same species. If the degree of hybridization is 25–70%, they are designated the same genus. If the hybridization is less than 25%, the test bacteria are deemed unrelated genera.

Such DNA–DNA hybridization assays using pure-culture microorganisms are clearly impossible to carry out with uncultured microorganisms – often characterized only by the sequence of 16S rRNA genes retrieved from a particular sample of soil, sediment, or water. When the 16S rRNA biomarker (sequence) is the primary measure of identity and taxonomy, the term OTU (for operational taxonomic unit) applies. The 16S rRNA gene-based OTUs can be grouped at a "species-like" level if their identities are at least 97% and as high as 98.7% identical (Box 5.2). Comparisons of 16S rRNA sequences among uncultured microorganisms should be viewed as an insightful, yet temporary and approximate, way to estimate taxonomy and phylogeny.

Whenever a microorganism becomes cultured (domesticated), the additional phenotypic and genotypic determinations are sure to refine prior "species" and "OTU" designations based solely on the 16S rRNA gene.

Of course, the ultimate means of genetic classification and comparison between microorganisms is the whole genome sequence (see Section 3.2 and Box 5.2). With whole genomes, everything is revealed. Systematic whole-genome sequence comparisons have demonstrated that cells of a common species share an average nucleotide identity (ANI) of 94–96% for protein-coding genes with a shared function (Box 5.2; Richter and Rosello-Mora, 2009). The ANI parameter, by definition, integrates all gene sequences across entire genomes. Yet traditional microbial taxonomists have given high weight to certain traits and low weight to others. In this approach, distinctions must be made between those genes encoding traits that are taxonomically and evolutionarily insightful and those that are not. Thus, choosing the right genes to efficiently assist in assembling taxa ("coherent groups of individuals that comprise species", see above) is still an active and challenging area of microbiology. Very likely, the future of microbial taxonomy and species designations will rely on sequence comparisons of many prudently selected genetic loci – a technique known as multilocus sequence analysis (MLSA; see Coenye et al., 2005; Gevers et al., 2005; Fraser et al., 2009; Rainey and Oren, 2011).

Science and the citizen

When taxonomic and genetic diversity really matters: *Escherichia coli* as a normal intestinal inhabitant and as a dangerous food-borne pathogen

Headline news from the United States Centers for Disease Control and your local newspaper

In the fall of 2006, packages of fresh spinach, grown in California and distributed nationally, were contaminated with *E. coli* O157:H7. Approximately 200 people in 26 states (Figure 1) were infected. Symptoms included severe sudden abdominal cramps and bloody diarrhea lasting from 1 to 8 days.

In 1993, a similar outbreak of painful, bloody diarrhea led to kidney failure in a large group of children in Seattle, Washington. Some of the children died. The illnesses were also caused by *E. coli* O157:H7, contracted from contaminated hamburgers sold to the public by a single fast-food restaurant chain.

Outbreaks of *E. coli* O157:H7 infection occur regularly and have been both large and small in localized areas and across several states. Transmission of food-borne *E. coli* was first associated with contaminated ground beef but has also been spread through: unpasteurized milk and fruit juices; spinach, lettuce, sprouts, salami, and contaminated drinking water; swimming in or drinking sewage-contaminated water; contact with infected animals (such as in petting zoos); and person

to person, especially among children in day-care centers. The way *E. coli* O157:H7 is transmitted changes over time, which is why the United States Centers for Disease Control work closely with state health departments to monitor and investigate cases and outbreaks of *E. coli* O157:H7.

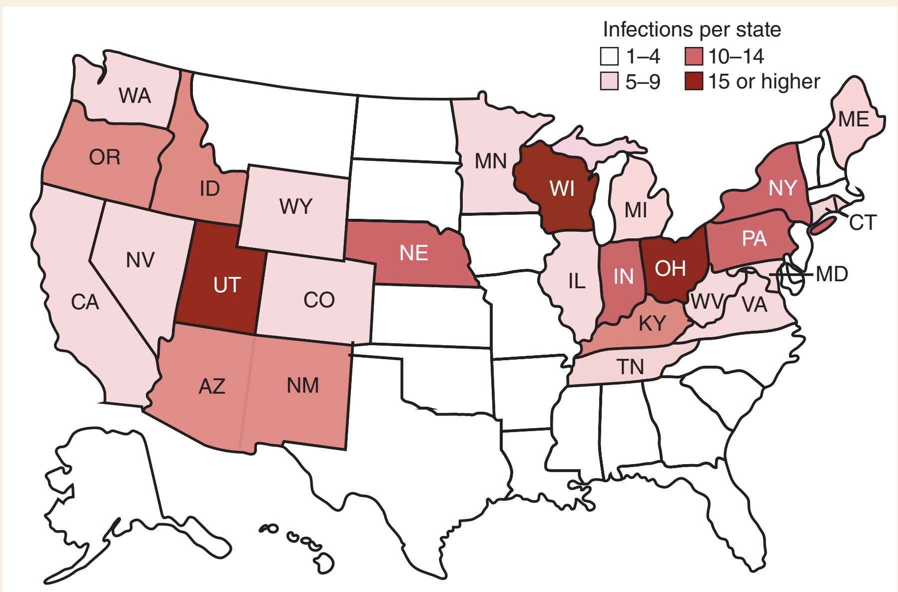


Figure 1 Occurrence of *E. coli* O157:H7 infections caused by spinach consumption in the United States, fall 2006. (From Centers for Disease Control, with permission, http://www.cdc.gov/foodborne/ecolispinach/100606.htm.)

SCIENCE: A harmless inhabitant of the human intestine evolves into a pathogen

Background

E. coli O157:H7 is one of hundreds of strains of the bacterium *E. coli*. Although most strains are harmless, strain O157:H7 produces powerful toxins that can cause severe illness. *E. coli* O157:H7 has been found in the intestines of healthy cattle, deer, goats, and sheep. *E. coli* O157:H7 was first recognized as a cause of human illness in 1982 during an outbreak of severe bloody diarrhea; the outbreak was traced to contaminated hamburgers. Since then, more infections in the United States have been caused by eating undercooked ground beef than by any other food.

How did E. coli O157:H7 develop?

Strain O157:H7's entire genome has been sequenced – as has the genome of the benign inhabitant of the human intestine *E. coli* strain K-12 (see Section 3.2). The pathogen's genome is encoded in 5.62 Mb of DNA, while strain K-12's genome is 4.74 Mb; 4.1 Mb is shared in common (Ohnishi et al., 2002). Much of the 1.5 Mb of DNA characteristic of strain O157:H7 carries virulence genes that encode factors for attachment to host cells, production of molecules that interfere with host signaling pathways, and production of two different types of toxins (Shiga toxins) that disrupt protein synthesis in the host. The bacterium also contains a

large virulence-associated plasmid. Approximately two-thirds of strain O157:H7's virulence genes are associated with virus-like genetic elements (Ohnishi et al., 2002). This strongly suggests that horizontal gene transfer is the mechanism that gradually converted the original benign *E. coli* into a powerful pathogen (see Sections 2.10, 8.5, 8.7, and 9.2 for additional discussion of mechanisms of evolution).

Evolutionary analysis has led to a model of strain O157:H7's development. Figure 2 shows a scheme of the likely final stages in a series of genetic acquisitions and deletions that led to the contemporary pathogenic *E. coli* strain O157:H7 Sakai.

A major lesson here is that microbial diversity is not necessarily reflected in the naming (the taxonomy) of a bacterium. Both strains K-12 and O157:H7 are E. coli. *However, hidden behind their common species designation are big differences, sometimes deadly ones.*

Research essay assignment

The genetic basis of some diseases in some pathogens (of plants, animals, humans) has been traced to "pathogenicity islands". These are large, unstable regions of a microorganism's chromosome that have been acquired through horizontal gene transfer (see Section 5.9). Encoded in the genes are biosynthetic pathways that lead to virulence factors that include cell surface structures, toxins, or other traits. Based on a survey of the scientific literature, write an essay defining pathogenicity islands and the role they play in at least one type of disease.

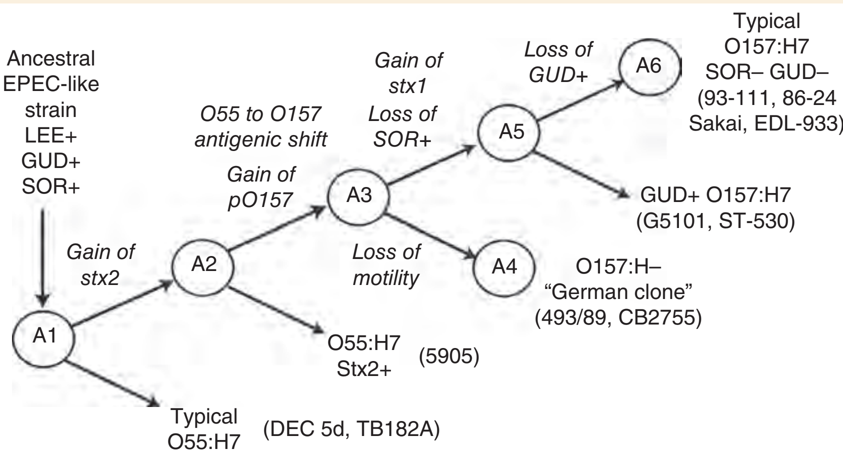


Figure 2 Stepwise genomic changes leading to the emergence of *E. coli* O157:H7. The model proposes six stages (A1–A6) for the evolution of *E. coli* O157:H7 from an enteropathogenic *E. coli*-like ancestor. "Gain" and "loss" refer to the acquisition and ejection (respectively) of DNA carrying clusters of genes encoding particular traits. EPEC, enteropathogenic *E. coli*; GUD, β-glucuronidase utilization; LEE, locus of enterocyte effacement; pO157, large virulence plasmid; SOR, sorbitol utilization; stx2 and stx1, Shiga toxins. (From Wick, L.M., W. Qi, D.W. Lacher, and T.S. Whittam. 2005. Evolution of genomic content in the stepwise emergence of *Escherichia coli* O157:H7. *J. Bacteriol.* **187**:1783–1791 and Feng, P., K.A. Lampel, H. Karch, and T.S. Whittam. 1998. Genotypic and phenotypic characterization of *Escherichia coli* O157:H7. *J. Infectious Dis.* **177**:1750–1753. American Society for Microbiology and University of Chicago Press, with permission; see also Eppinger et al., 2011.)

5.4 PROCEEDING TOWARD CENSUS TAKING AND MEASURES OF MICROBIAL DIVERSITY

Now we return to the task begun in Section 5.2.

- **How do we take a census that documents the number and diversity of microorganisms in a natural habitat?**

While all four broad methodologies described in Box 5.1 can contribute information to a census, it is nucleic acid biomarkers, especially DNA sequences encoding the small subunit rRNA gene, that are widely recognized as the procedure of choice for conducting a census of microorganisms that occur in nature. Small subunit rRNA genes can be *sampled*, and they afford both *recognition* and *classification* (see Sections 5.2 and 5.3) of the prokaryotic rRNA (16S) and eukaryotic (18S) hosts of the genes. Recovering rRNA gene sequences from soil or sediments is like recovering license plates from an automobile wrecking yard – even if the automobile is lost in the heap, the automobile may be accurately accounted for by its license plate. Several insightful reviews and recent methodological updates on assessing microbial diversity and taking a census have appeared (Hughes et al., 2001; Ward, 2002; Bohannan and Hughes, 2003; Schloss and Handelsman, 2004, 2006; Curtis and Sloan, 2005; Haegeman et al., 2013; Caporaso et al., 2010; Bunge 2009; Zinger et al., 2012; Schloss et al., 2009; Schloss and Westcott, 2011; Prosser et al., 2007; Pedrós-Alió, 2006). These are the basis for the following discussion and the summary on diversity measures shown in Box 5.3.

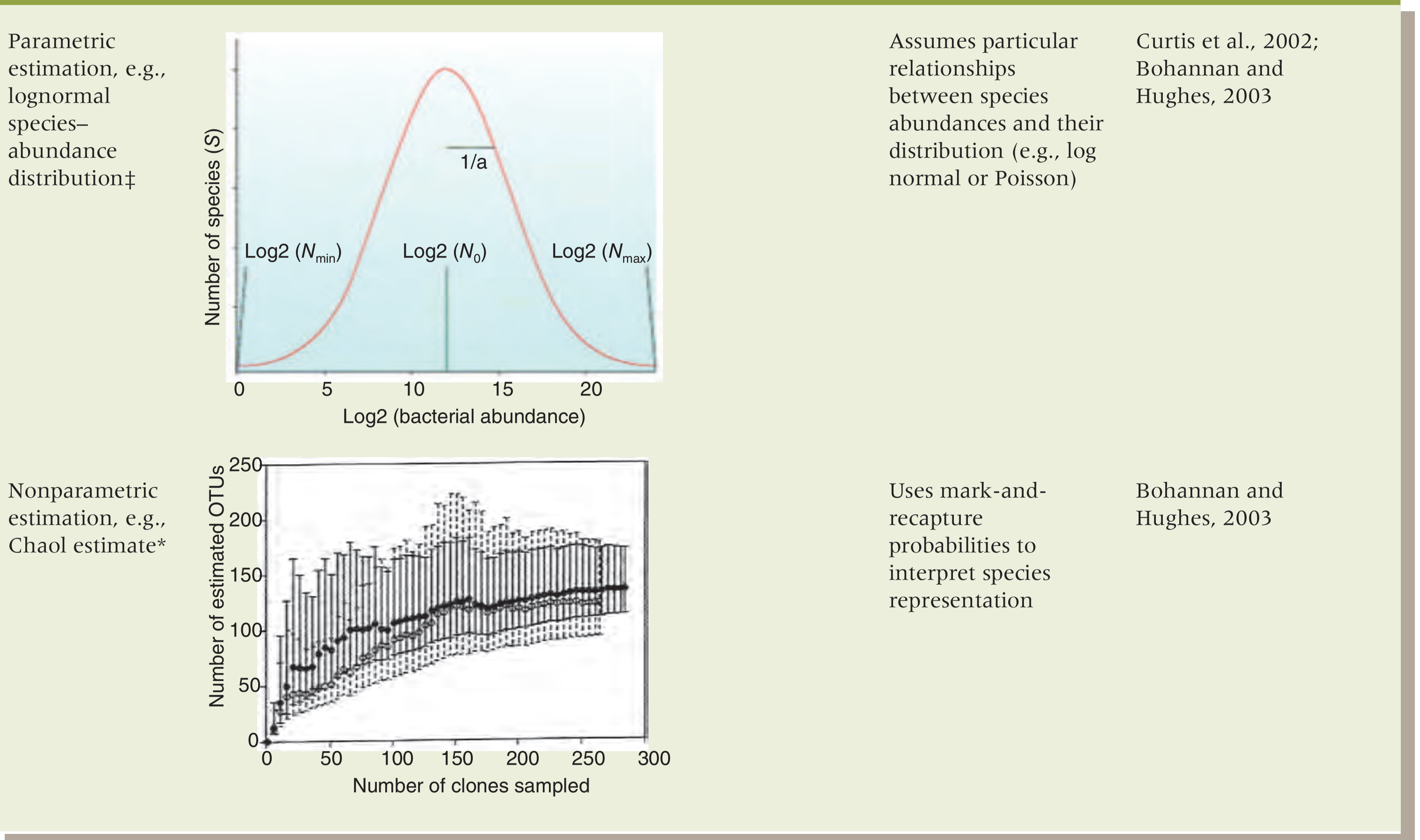
Rarefaction curves

A rarefaction curve approach to microbial diversity (a common one in plant and animal ecology) measures the accumulation of distinctive taxa as a function of sampling effort. This approach presumes that, as sampling proceeds, members of the rarest species will gradually be added to the total (Box 5.3). Thus, the asymptote in the rarefaction accumulation curve estimates total diversity in the habitat sampled. When Tringe et al. (2005) sampled 16S rRNA genes from soil, the pool of 1700 clones revealed 847 sequence types from more than a dozen far-ranging taxonomic phyla. Moreover, the rarefaction curve built from the data failed to indicate an asymptote. Likewise, when Elshahed et al. (2008) obtained 13,001 near full-length 16S rRNA gene sequences from a single soil sample in Oklahoma, the rarefication curve at the 3% similarity cut-off showed no hint of a plateau. Thus, the data from both Tringe et al. (2005) and Elshahed et al. (2008) (like the rarefaction data in Box 5.3) did not approach a complete count of sequence types. This means that microbial diversity was very high and that it was incompletely sampled. As will become clear here, and in

Box 5.3

Estimating microbial diversity: let me count the ways

Diversity estimation approaches	Example	Comments	References
Rarefaction curves*	Number of OTUs observed (0–90) vs. Number of clones sampled (0–300)	Counts species as a function of sampling effort Compares observed richness among sites, treatments, or habitat that are unequally sampled	Hughes et al., 2001; Bohannan and Hughes, 2003; Schloss and Handelsman, 2004
DNA hybridization†	Reassociated % (−10–70) vs. Log C_0t moles x sec x L^{-1} (−2.0–4.0); Soil; *E. coli*	Rates of DNA–DNA reassociation are inversely proportional to species diversity	Torsvick et al., 1990; Øvreås et al., 1998; Gans et al., 2005



Parametric estimation, e.g., lognormal species–abundance distribution‡		Assumes particular relationships between species abundances and their distribution (e.g., log normal or Poisson)	Curtis et al., 2002; Bohannan and Hughes, 2003
Nonparametric estimation, e.g., Chaol estimate*		Uses mark-and-recapture probabilities to interpret species representation	Bohannan and Hughes, 2003

Box 5.3 *Continued*

Diversity estimation approaches	Example	Comments	References
Phylogenetic trees§		The diversity of sequences graphically depicted in phylogenetic trees is reflected in the number of taxa (leaves of a tree), branch lengths, and branching patterns	Bohannan and Hughes, 2003; Robertson et al., 2005
Ecological diversity indices, e.g., Shannon Index	$H = -\sum_{i=1}^{s}(pi)(\log_2)(pi)$ Assumes random sampling from large community in which total species number is known	H = index of species diversity; S = number of species; pi = proportion of total sample belonging to the ith species	Atlas, 1984; Krebs, 2001; Haegeman et al., 2013

*Figure from Hughes, J.B., J.J. Hellmann, T.H. Ricketts, and B.J.M. Bohannan. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol*. 67:4399–4406. With permission from the American Society for Microbiology.

†Figure from Øvreås, L., S. Jensen, F.L. Daae, and V. Torsvik. 1998. Microbial changes in a perturbed agricultural soil investigated by molecular and physiological approaches. *Appl. Environ. Microbiol*. **64**:2739–2742. With permission from the American Society for Microbiology.

‡Figure reprinted from Bohannan, B.J.M. and J. Hughes. 2003. *Curr. Opin. Microbiol.* 6. New approaches to analyzing microbial biodiversity data. *Curr. Opin. Microbiol*. **6**:282–287. Copyright 2003, with permission from Elsevier.

§Figure modified from Morrell, V. 1997. Microbial biology: microbiology's scarred revolutionary. *Science* **276**:699–702. Reprinted with permission from AAAS.

Sections 6.7, 6.9, and 6.10, incomplete sampling is the rule when studying naturally occurring microbial communities.

There is, however, new evidence suggesting that rarefication curves of 16S rRNA genes from soil microbial communities may plateau after millions of sequences have been obtained (e.g., using Illumina-based technology; Bartram et al., 2011; see Section 6.10).

DNA hybridization

DNA–DNA reassociation rates are another way to estimate community diversity. DNA can be extracted from environmental samples and after the strands of the double helix are denatured at high temperature, their rate of reassociation (or reannealing) is determined by the size and complexity of the DNA. Torsvick et al. (1990) reasoned that pooled genomic DNA from soil might abide by the same rehybridization rules as large genomic DNA from purified cultures (Box 5.3). The data from Torsvick et al. (1990) suggested that soil DNA reassembled so slowly that it was 7000 times as complex as the genome of a single bacterium. Subsequent estimates of species diversity in soil by Torsvick et al. (2002) suggest there are 40,000 species per gram.

Diversity estimation theory was taken even further by Gans et al. (2005), who reinterpreted DNA reassociation curves from soil microbial communities. The authors recast equations describing DNA annealing rates to allow quantitative comparison of different ecological species–abundance models. The analysis showed that a power law best described the abundance distribution of prokaryotes. The authors concluded that more than one million genomes occurred in pristine soil – exceeding previous estimates by two orders of magnitude.

Parametric estimation

An example of parametric diversity estimation procedures are those used by Curtis et al. (2002), who drew upon ecological theory to devise a way to calculate total diversity in a microbial community. Standard measures of biotic diversity in plant and animal ecology rely upon two fundamental pieces of information: the number of species and the number of individuals in each species. Such data are often presented as "species–abundance curves", in which the number of species is plotted versus the number of individuals per species. Curtis et al. (2002) postulated that, by assuming a particular type of statistical distribution (lognormal), species–abundance curves apply to microorganisms in nature (Box 5.3). The investigators were able to relate the total diversity of prokaryotic communities to the ratio of two potentially measurable variables: the total number of individuals and the abundance of the most abundant species. Using this approach, Curtis et al. (2002) reported the number of species in ocean water was 160 per milliliter; in soil the species abundance was 6400–38,000 per gram.

Nonparametric estimation

A nonparametric estimation of the microbial diversity, the Chao1 statistic, requires no assumptions about species distribution models (Box 5.3). Instead, mark–release–recapture statistics are used. These were developed for estimating the size of animal populations. The approach documents the proportion of species (or OTUs) that have been observed before (recaptured), relative to those that have been observed only once. Samples from diverse communities are predicted to contain few recaptures.

Chao1 estimates total species richness as:

$$S_{Chao1} = S_{obs} + n_1^2 / 2n_2$$

where S_{obs} is the number of observed species, n_1 is the number of singletons (species captured once), and n_2 is the number of doubletons (species captured twice).

The Chao1 statistic estimates both the total diversity of a given habitat and the precision of the estimates (Bohannan and Hughes, 2003), thus facilitating comparison of diversities between habitats. The index is well suited for data sets skewed toward low-abundance species, as is likely for microbial communities (Hughes et al., 2001).

Phylogenetic trees

Phylogenetic trees are routinely prepared as a graphic way to display contrasts between sequences of nucleic acid biomarkers (especially the genes encoding small subunit rRNA; see Box 5.3). After alignment of the sequences, a variety of computer-based software algorithms (see Sections 5.5 and 5.7) can precisely assess the degree of relatedness between sequences. The results are dendrograms (or phylogenetic trees) in which closely related sequences appear as neighboring "leaves" or "clusters of twigs", while unrelated sequences are placed on far-removed branches. The linear distances between any two positions on the tree are proportional to their degree of sequence dissimilarity. The branching patterns of a given phylogenetic tree display distinctive identities of the taxa. Branching patterns can be qualitatively and quantitatively compared between trees (Martin, 2002; Bohannan and Hughes, 2003; Robertson et al., 2005; Caporaso et al., 2010; Schloss et al., 2009).

Ecological diversity indices

Ecological diversity indices have been devised by ecologists studying plants and animals, constituting a wide variety of species–diversity measures (Atlas, 1984; Atlas and Bartha, 1998; Krebs, 2001). These

include richness, dominance (Simpson Index), and equitability (Shannon Index) indices (see Box 5.3). Many of the calculated parameters make presumptions (e.g., random sampling, complete knowledge of total species, and particular statistical species–abundance distributions) that some microbial ecologists feel are unlikely to apply to naturally occurring microbial communities. However, Haegeman et al. (2013) have recently re-examined Shannon and Simpson indices as versions of the more general set of "Hill diversity" measures. The verdict is "Shannon and Simpson diversity indices provide a robust way to quantify and compare microbial diversity".

Comparative approaches

Hong et al. (2006) adopted an integrated approach to compare the effectiveness of four parametric and two nonparametric procedures for estimating species richness using a single 16S rRNA gene library from marine sediment. Their data consisted of >500 clones and >400 OTUs. Hong et al. (2006) argued that the validity of all prior estimation approaches was questionable. The investigators applied consistent, rigorous criteria (such as statistical "goodness-of-fit" and minimized standard error) to all six statistical models. The study selected the "Pareto" parametric model as the most powerful means to evaluate their data set; the model estimated the total diversity of the marine sediment to be 2434 ± 542 bacterial species. Schloss and Handelsman (2006) used 1033 16S rRNA gene sequences from an Alaskan soil to choose the best model for simulating microbial diversity. These investigators found that a "truncated log normal frequency distribution" best simulated the data and that in order to capture a total diversity of 5000 OTUs with 95% confidence, 400,000 16S rRNA genes would need to be sequenced (Schloss and Handelsman, 2006).

Note that census taking will be discussed further in Sections 6.7, 6.9, and 6.10. All but one (DNA–DNA hybridization) of the approaches mentioned above and in Box 5.3 for assessing microbial diversity have been incorporated into the key software packages (e.g., QIIME and MOTHUR; Caporaso et al., 2010; Schloss et al., 2009) routinely used to analyze and compare the composition of microbial communities based on sequences of extracted nucleic acids.

5.5 THE TREE OF LIFE: OUR VIEW OF EVOLUTION'S BLUEPRINT FOR BIOLOGICAL DIVERSITY

Human knowledge of the diversity of life forms has undergone at least four major revolutions. In 1866, Haeckel presented a three kingdom view of plants, protists (single-celled eukaryotes), and animals. In 1937,

Chatton recognized that fundamental cellular architecture justified only two main kingdoms: prokaryotes and eukaryotes. In the late 1950s, Whittaker again used phenotypic traits to develop the five kingdom model: bacteria, fungi, protists, plants, and animals. In the 1970s, C.R. Woese (Figure 5.4) and colleagues devised a far more direct and quantitative way to discover relationships between forms of life. These researchers realized that genotypic information (e.g., the sequencing of proteins and nucleic acids) is far superior to phenotypic information for discovering evolutionary relationships. *Phenotypic comparisons are made based on subjective judgments; while sequences of nucleotides or amino acids can be translated into objective, precise, mathematically defined measures of true phylogenetic relatedness* (Woese, 1987).

Woese and colleagues sought a *molecular chronometer* that would reveal evolutionary relationships between life forms. Molecular chronometers exhibit sequence changes, caused by random mutations, that tick away (ideally at a constant rate) like the second hand of a clock. The amount of sequence change in the molecule carried by two ancestrally related lineages (species) is termed *sequence divergence*. The degree of divergence between the two lineages reflects both the rate that mutations are fixed in the molecule and the time over which the mutational changes have occurred. To be useful in molecular phylogeny, the chronometer of choice must: (i) have clock-like random mutations; (ii) change at rates commen-

Figure 5.4 C.R. Woese, molecular microbiologist at the University of Illinois, whose phylogenetic analyses led to the three domain tree of life. (From University of Illinois at Urbana-Champaign, with permission.)

surate with the evolutionary distance of interest; and (iii) be rich enough in information to allow fine distinctions to be made, while maintaining stability in overall structure. Woese and colleagues realized that rRNAs (especially small subunit rRNAs) meet the criteria for an ideal molecular chronometer: (i) they occur in all cellular organisms that carry out protein biosynthesis; (ii) they feature a high degree of functional constancy; (iii) despite functional constraints, different positions in the three-dimensional structure of rRNAs change at different rates – allowing both distant and close phylogenetic relationships to be charted; (iv) their size offers at least 50 helical stalks that constitute a rich source for comparative analysis; and (v) the genes that encode rRNAs can be sequenced relatively easily (Woese, 1987).

Figure 5.5 shows secondary structures of small subunit rRNA molecules representative of life's three domains. Each of the domains (*Bacteria* and *Archaea* carry 16S rRNA; *Eukarya* carry 18S rRNA) exhibits common resemblances and characteristic differences. To illustrate sequence- and structural-level differences that afford relatively fine distinctions to be drawn, consider Figure 5.6, which focuses upon two helical domains in the bacterial 16S rRNA molecule that help define α, δ, and β/γ subdivisions of the Proteobacteria (this taxonomic class will be discussed in Section 5.6). A major distinction between the δ-Proteobacteria (Figure 5.6, right) and the other three subdivisions is a 10 base pairs (bp) extension in the stalk of the downward-pointing helix. Another clear distinction in the structures is in the left-pointing helix.

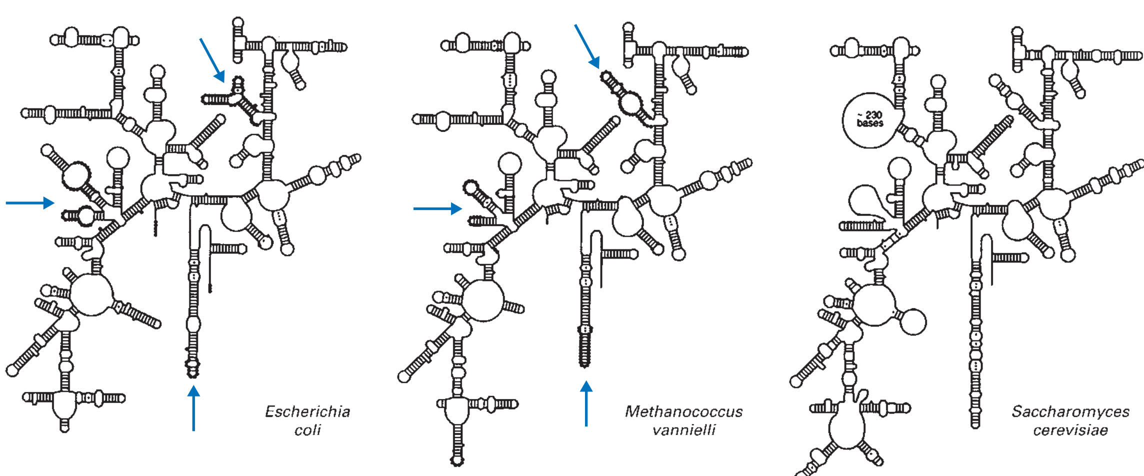


Figure 5.5 Representative small subunit rRNA secondary structures for the three primary domains: from left to right, *Bacteria*, *Archaea*, and *Eukarya*. Arrows pointing within the *Escherichia coli* and *Methanococcus vannielli* structures identify three key locales where *Bacteria* and *Archaea* characteristically differ. (From Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271. With permission from the American Society for Microbiology.)

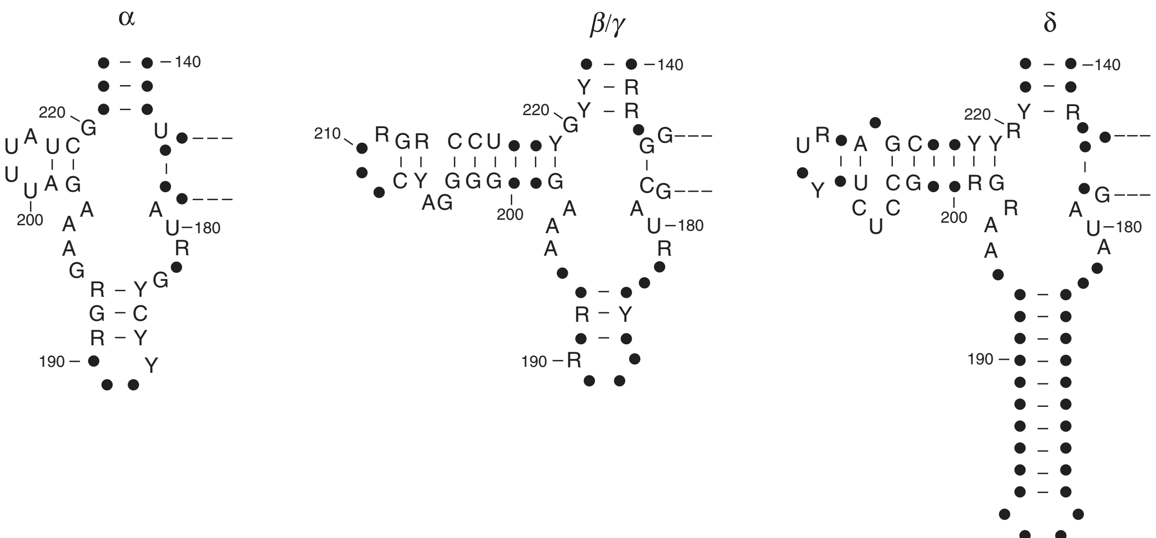


Figure 5.6 Comparison of the secondary structures of two helical domains of rRNA for three subdivisions of the Proteobacteria – dots are shown in locations of nucleotides that are not highly conserved. (From Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev*. **51**:221–271. With permission from the American Society for Microbiology.)

The stalk of this helix consists of 2 bp in the α-Proteobacteria and 8 bp in the β/γ- and δ-Proteobacteria.

Using structural molecular contrasts described above and computer-based algorithms that compare the sequence of genes encoding small subunit rRNA molecules, Woese and colleagues created a tree of life that displays true, evolution-based phylogenetic relationships among organisms (Figure 5.7). The power of molecular phylogenetic techniques cannot be overstated. Life consists of three major domains (*Bacteria*, *Archaea*, and *Eukarya*) and many of the lineages at the base of the tree are adapted to high temperatures (hyperthermophilic), suggesting that the last universal common ancestor (see Section 2.7) emerged from a hot primeval habitat and/or adapted to hyperthermophilic conditions shortly after emerging (see Section 2.7).

Implicitly, the three-domain concept supersedes and negates prior historical paradigms in biology (three kingdoms, prokaryote/eukaryote, and five kingdoms; mentioned above). *Archaea* are distinctive from *Bacteria* (they are like night and day). Grouping these two domains within a single term, "prokaryote", is considered by many microbiologists to be unwise and erroneous (Doolittle and Zhaxybayeva, 2013). Despite this fact, "prokaryote" has a utilitarian aspect that is likely to allow it to linger in the microbiological lexicon for many years into the future.

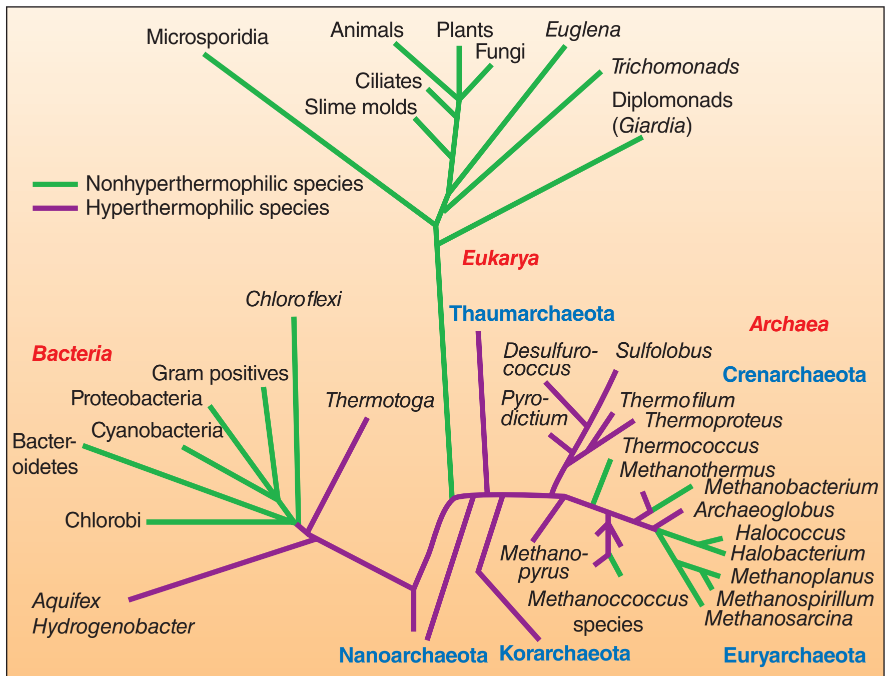


Figure 5.7 Tree of life based on small subunit rRNA sequence analysis. Phyla residing at the base of the tree (in purple) are thermophiles. Phyla evolving later (in green) are nonthermophilic. More detailed taxonomic subgroups (class, order, family, genera, and species) are not shown. (Modified from Morrell, V. 1997. Microbial biology: microbiology's scarred revolutionary. *Science* **276**:699–702. Reprinted with permission from AAAS.)

5.6 A SAMPLING OF KEY TRAITS OF CULTURED MICROORGANISMS FROM THE DOMAINS *EUKARYA*, *BACTERIA*, AND *ARCHAEA*

Early evolutionary events on Earth were presented in Chapter 2, which summarized current ideas and facts regarding stages of development in early life: prebiotic Earth, the iron/sulfur world, the RNA world, the "last universal common ancestor", early division of *Bacteria* from *Archaea*, the rise of oxygen, endosymbiotic theory, and the emergence of *Eukarya*. The long series of evolutionary and biogeochemical successions has delivered to us the contemporary tree of life (Figure 5.7). Woesean analysis of small subunit rRNA molecules produced both the broad three-domain tree of life and the many major divisions (phyla) with finer subdivisions within each domain. The branches, twigs, stems, and leaves on the tree (not shown in Figure 5.7) roughly correspond to the progressively refined taxonomic categories of class, order, family, genera, and species (see Table 5.1 and

Section 5.3). The version of the tree of life shown in Figure 5.7 portrays phylogenetic and taxonomic relationships between representative *cultured members* of all three domains. Because the cultured *Bacteria*, *Archaea*, and *Eukarya* shown in Figure 5.7 have been subjected to phenotypic, genotypic, and ecological analyses, a great deal is known about their biology. We now survey below the key phenotypic traits of selected members of the three domains.

The domain *Eukarya* (protists and the fungi)

Several fundamental structural, cellular, genetic, and phenotypic differences between eukaryotes and prokaryotes were presented in Chapter 2 (see Section 2.12, Table 2.3, and Box 2.3). Eukaryotic microorganisms often feature larger cell sizes (10–25 μm versus 1 μm of prokaryotes), organelles (e.g., nucleus, mitochondria, chloroplasts, plastids, Golgi bodies), multicellularity, tissue development, flexible cell walls, complex sexual reproduction involving gametes produced by meiosis, and a high degree of metabolic specialization (an absence of metabolic versatility). The term "protists" applies to all eukaryotic microbial life, except the fungi. In surveying branches on the tree of life (see Figure 5.7), it is clear that maximum temperatures for *Eukarya* (generally ~50–60 °C) fall far below the extreme temperature adaptations (>80 °C) exhibited by prokaryotes. Furthermore, unicellular *Eukarya* (diplomonads, microsporidia, trichomonads, slime mods, ciliates, euglena) appear to have emerged early in evolution – they reside at the base of the *Eukarya* trunk. Fungi, plants, and animals emerged last in evolution. The reader may recall that the early, phenotype-based (and anthropocentric) versions of the tree of life incorrectly placed plants, animals, and fungi in their own taxonomic kingdoms. In terms of evolutionary distance gauged by branch lengths in the small subunit rRNA tree, the distinctions between animals, plants, and fungi are minor when compared to the branch lengths of other phyla within and between domains.

The taxonomy of cultivated microbial eukaryotes is intricate and still surprisingly unsettled. Historically, the identification and classification of protists and fungi have relied upon microscopy – first light microscopy and later electron microscopy. It has been discovered that phylogenetic trends revealed by small subunit rRNA genes (Figure 5.7) are often not corroborated by other highly conserved genes (e.g., genes encoding RNA polymerase, ATP synthase, heat shock proteins). Moreover, nutritional phenotypes (e.g., heterotrophy versus phototrophy) and morphological traits (amoeboid versus flagellated forms) have sometimes been proven to be misleading criteria for establishing relatedness among protists. Also, lack of mitochondria in some protists (diplomonads and trichomonads) was once taken as evidence of an early-branching, "primitive" life style – but the current view is that the evolutionary path taken by these organisms may have involved loss of function; thus, such organisms may have appeared

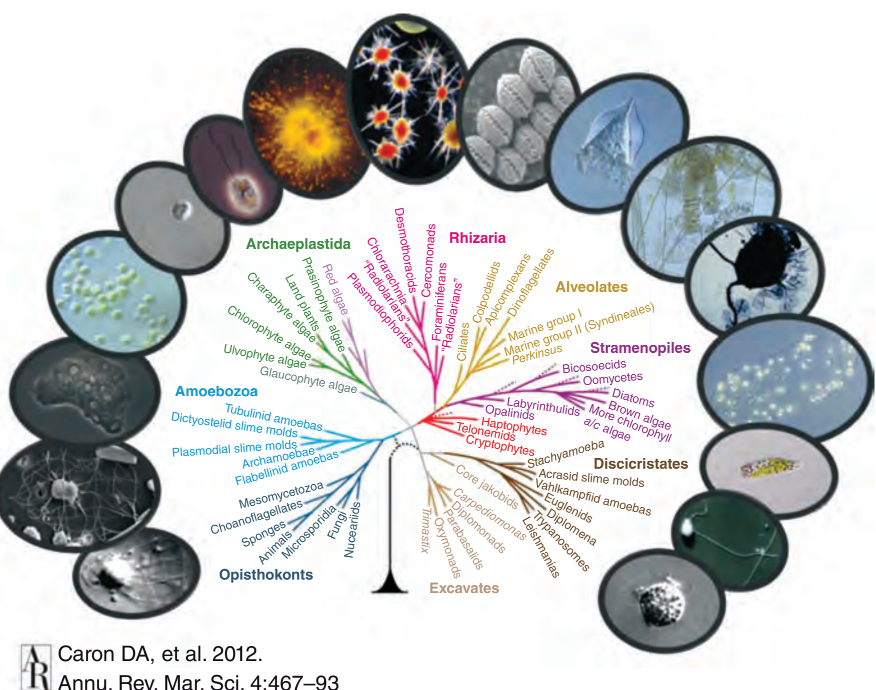


Figure 5.8 A recent depiction of the eight major branches (supergroups) of the phylogenic tree for protists and fungi. Single-celled species (protists) occur in every supergroup within the domain *Eukarya* and constitute the entirety of many of them. Here, the recent phylogenetic scheme (Caron et al., 2012) is illustrated with micrographs of common taxa. The images depict: (*clockwise from lower left*) two micrographs of choanoflagellates, a free-living lobose amoeba, minute chlorophyte algae, the prasinophyte *Pyramimonas*, the heterotrophic cercozoan flagellate *Cryothecomonas*, the planktonic foraminiferan *Orbulina*, a mixed natural assemblage of *Acantharia*, the photosynthetic dinoflagellate *Alexandrium*, a tintinnid ciliate, a mixed diatom assemblage, the heterotrophic chrysomonad *Paraphysomonas*, the colonial haptophyte *Phaeocystis*, the euglenid flagellate *Eutreptiella*, a heterotrophic bodonid flagellate, and a heliozoan, *Alexandrium*. (From Caron, D.A., P.D. Countway, A.C. Jones, D.Y. Kim, and A. Schnetzer. 2012. Marine protistan diversity. *Annu. Rev. Mar. Sci.* **4**:467–493. Reprinted with permission from Annual Reviews.)

late in evolution. Recent reviews on the biology and phylogeny of both fungi and protists include those by Hibbett and Taylor (2013), Adl et al. (2005, 2007), Caron et al. (2012), Pawlowski et al. (2012), Pace (2009), Keeling et al. (2005), and Hampl et al. (2009). The current, detailed view of the phylogeny of single-celled eukaryotes spans eight major supergroups, as described by Caron et al. (2012) (Figure 5.8). Below is a brief description of each of the eight supergroups named in Figure 5.8 for recent phylogeny of the single-celled *Eukarya*. Traits based on more traditional

taxonomic groupings (i.e., nutrition, morphology, and/or pigments for algae, fungi, protozoa, and slime molds shown in Figure 5.7) will follow.

Opisthokonta

The opisthokonts (Figure 5.8) are, evolutionarily, the highest eukaryotic supergroup. Regarding protistan members, this group contains the "nucleariid" amoebae and a class of small, free-living, heterotrophic flagellates, which can be important bacterivorous species in a variety of ecosystems. These protists share the morphological feature of a single posterior flagellum on motile cell stages. The opisthokonta supergroup also contains the animals and the fungi.

Amoebozoa

Amoebozoans (Figure 5.8) include many types of the amoeboid forms of life that are abundant in soils and sediments (both freshwater and marine). Amoebae have highly flexible cells and use pseudopodia for motility on surfaces and for feeding via phagocytosis. Included here are "lobose amoebae", along with the slime molds (common in terrestrial soils), as well as the parasitic entamoebae.

Archaeplastida (Plantae)

This supergroup (Figure 5.8) contains ecologically important green and red algae (both multicellular and single-celled forms). A commonality here is intracellular plastids (i.e., chloroplasts and related photosynthetic organelles). This supergroup includes vascular land plants.

Rhizaria

The Rhizaria supergroup (Figure 5.8) is extremely diverse, phenotypically. It includes some colonial protists that are large enough to be distinguished by the human eye, as well as microscopic organisms that are heterotrophic and/or photosynthetic. Skeletal structures of silica, calcium carbonate, and strontium sulfate are produced by radiolaria, foraminifera, and acantharia, respectively; these can be of immense geological and paleontological importance. Regarding phylogenetic markers of this group, amino acid insertions into the eukaryotic housekeeping regulatory protein, polyubiquitin, are shared among members of this group.

Alveolata and Stramenopila (two large and related supergroups, sometimes clustered together as "Chromalveolata")

These groups (Figure 5.8) represent a broad range of well-recognized taxa that are ecologically significant. "Alveoli" are a structural trait shared by all members of the Alveolata. Alveoli are membrane-bound cavities on the

inner surface of the plasma membranes of the cells of these protists. Among key alveolates are dinoflagellates and cilates, and parasitic forms that feature unique cellular structures that are the basis for the "apicomplexan" category within the protists.

Stramenopiles (sometimes called "heterokonts") also share a common, yet distinctive cellular structure: two different types of flagella, with one flagellum bearing "mastigonemes" that have a tripartite structure. A broad diversity of stramenopiles includes the familiar (diatoms, golden algae, and brown algae) and the unfamiliar (bicosoecids and the oomycetes, or water molds).

Excavata and Discicristates (two large and related supergroups sometimes labeled simply as "Excavata")

These supergroups are formed, respectively, from two large branches in the protistan tree (Figure 5.8). Represented are highly diverse organisms from the point of view of nutrition, lifestyle, and ecological niche. Some of the "Excavata/Discicristates" group are parasitic in animals, but many are ecologically important free-living taxa active in aquatic habitats. The euglenozoa include protistan species that are photosynthetic, heterotrophic, or mixotrophic flagellates. The excavates also include diplomonads and parabasalids (dwelling in anoxic habitats, such as animal intestines, either symbiotically or as parasites); they lack mitochondria, but feature other organelles (hydrogenosomes or mitosomes that catalyze ATP generation via fermentation).

Traditional classification of single-celled Eukarya

While information in Figure 5.8 and the above-associated text is accurate and up-to-date, it is wise and practical to be familiar with more traditional terminology and classification of single-celled *Eukarya*. Below is a more traditional portrayal of protistan and fungal biotic diversity. Table 5.2 provides an overview of the key traditional traits of eukaryotic microorganisms.

Algae

Algae are water-dwelling, oxygen-evolving phototrophs that can be unicellular or multicellular. Figure 5.9 shows a photomicrograph of typical algal cells. All algae have single-cell reproductive structures. Evolutionarily, algae appear to be the ancestor of land plants – the latter have developed sophisticated vascular systems and reproductive structures that lessen their dependence upon free water for growth, buoyancy, and reproduction. With the exception of oceanic brown algae (known as kelp), algae are structurally far less complex than land plants. Chlorophyll *a* is the pivotal photosynthetic pigment used in the chloroplasts of all algae. Traditional taxonomic divisions within the algae rely upon: accessory photosynthetic pigments (e.g., carotenoids and phycobilins), morphology, life cycle, food reserve materials (such as polysaccharides), and ecology. Table 5.3 provides

Table 5.2

Broad overview of eukaryotic microorganisms and their traits. (Compiled and modified from Madigan and Martinko, 2006)

Group	Nutrition	Motility	Structure	Reproduction
Fungi	Osmotrophic (uptake of soluble nutrients through cell wall)	Nonmotile (except some spores)	Filamentous, coenocytic (unicellular), mitochondria	Complex patterns of haploid, diploid, dikaryotic, and/or heterokaryotic states
Protists				
Algae	Photosynthetic	Flagella (amoeboid)	Unicellular, filamentous, multicellular, mitochondria and chloroplasts	Sexual, asexual; alternation of generations – haploid and diploid stages
Slime molds (Amoebozoa)	Phagotrophic	Amoeboid	Coenocytic, unicellular; mitochondria	Haploid, diploid
Protozoa: ciliates, amoebae, *Euglena*, trichomonads, diplomonads, microsporidia	Phagotrophic, osmotrophic, photosynthetic	Flagella, cilia, amoeboid	Unicellular, complex mixture of evolutionarily advanced cells (bearing mitochondria and chloroplasts) and simpler cells (mitochondria-free), featuring, instead, hydrogenosomes or mitosomes)	Complex patterns of haploid and diploid states

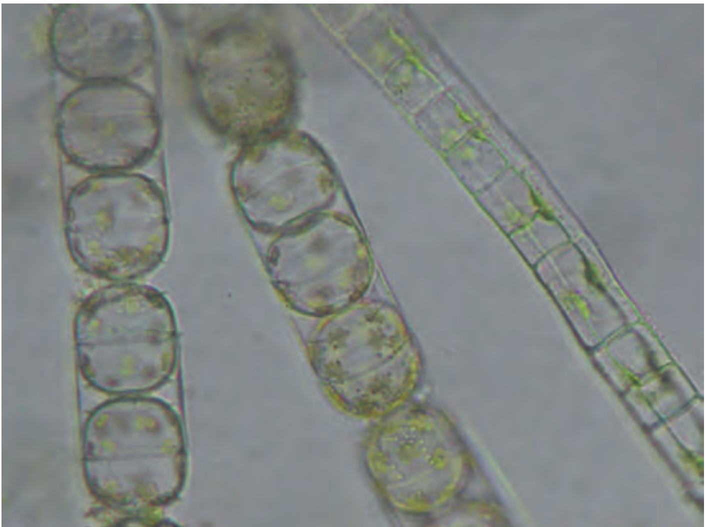


Figure 5.9 Image of the green alga, *Melosira*, found in a salt marsh, Heron's Head Park, San Francisco, ×800 magnification. (From W. Lanier, with permission.)

Table 5.3

Properties of the major traditional groups of algae. (Modified from Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 475. Prentice Hall, Upper Saddle River, NJ. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ)

Traditional algal group	Taxonomy based on Figure 5.8 phylogeny	Common name	Morphology	Pigments	Typical representative	Carbon reserve materials	Cell wall	Major habitats
Chlorophyta	Archaeplastida	Green algae	Unicellular to leafy	Chlorophylls *a* and *b*	*Chlamydomonas*	Starch (α-1,4-glucan), sucrose	Cellulose	Freshwater, soils, a few marine
Euglenophyta	Discicristates (Euglenid)	Euglenoids	Unicellular, flagellated	Chlorophylls *a* and *b*	*Euglena*	Paramylon (β-1,2-glucan)	No wall present	Freshwater, a few marine
Dinoflagellata	Alveolate	Dinoflagellates	Unicellular, flagellated	Chlorophylls *a* and *c*, xanthophylls	*Gonyaulax*, *Pfiesteria*	Starch (α-1,4-glucan)	Cellulose	Mainly marine
Chrysophyta	Stramenopile	Golden-brown algae, diatoms	Unicellular	Chlorophylls *a* and *c*	*Nitzschia*	Lipids	Two overlapping components made of silica	Freshwater, marine, soil
Phaeophyta	Stramenopile	Brown algae	Filamentous to leafy, occasionally massive and plant-like	Chlorophylls *a* and *c*, xanthophylls	*Laminaria*	Laminarin (β-1,3-glucan) mannitol	Cellulose	Marine
Rhodophyta	Archaeplastida	Red algae	Unicellular, filamentous to leafy (coralline)	Chlorophylls *a* and *d*, phycocyanin, phycoerythrin	*Polysiphonia*	Floridean starch (α-1,4-glucan and α-1,6-glucan)	Cellulose ($CaCO_3$)	Marine
Xanthophyta	Stromatolite	Yellow-green algae	Unicellular, amoebae, coenocytic filaments	Chlorophyl *c*	*Vaucheria*	Leucosin	Cellulose, pectin (silica)	Freshwater

a summary of the properties of the seven traditional major groups of algae. Algae play key roles as primary producers (via photosynthesis) in the aquatic habitats where they reside. In addition to the free-living photosynthetic lifestyle, some algae form symbiotic associations with other forms of life (see Section 8.1). These associations include lichens, coral reefs (where the density of unicellular dinoflagellates may be 30,000/ml), marine sponges, protozoa, flatworms, and mollusks (nudibranchs) that become photosynthetic after consuming algae.

A comprehensive treatment of algae is beyond the scope of this chapter. For additional information, see Gualtieri and Barsanti (2006), Wehr and Sheath (2003), Larkum et al. (2003), Graham and Wilcox (2000), Sze (1998), Stevenson et al. (1996), Raven et al. (2013), and Chaudhary and Agrawal (1996).

Fungi

Fungi are filamentous (tube-dwelling), nonphotosynthetic osmotrophs that contribute significantly to heterotrophic metabolism, especially to the cycling of plant biomass carbon, in many ecosystems. The three generally recognized morphological types for fungi are yeasts (unicellular), molds (surface coatings), and mushrooms (macroscopic fruiting bodies). Figure 5.10 shows a photograph representative of tree-dwelling fungi. Fungi possess cell walls, often composed of chitin (a polymer of *N*-acetyl-glucosamine also found in the exoskeletons of insects and crustaceans). At the cellular level, the tubular walls that house the cytoplasm, nucleus, and other organelles form filaments that are known as hyphae. “Coencoytic” fungi are those whose hyphae lack regular partitions (septa) that divide the cytoplasm into uninucleate cells. The occurrence of septa and their morphology are traditionally key distinguishing traits in fungal taxonomy. A network of hyphae constitutes a mycelium. Furthermore, a large mass of mycelia is termed a thallus. Terrestrial fungi exist largely as extensive microscopic hyphal networks that penetrate large volumes of soil. Mushrooms (thalli) that appear on the soil surface are fruiting bodies – specialized structures for the dissemination of fungal propagules known as spores. The filamentous nature of many fungi, combined with biochemical equipment (enzymes) for digesting plant cell walls, allow them to be successful as plant pathogens and as heterotrophs that digest the biomass of cellulose- and lignin-rich fallen trees (see Section 7.3). Fungi also infect humans – causing medical mycoses that include opportunistic diseases of the skin, lungs, and other tissues. Life cycles and sexual reproduction in fungi may be highly complex, including processes such as: (i) dikaryotization, where mycelia of different strains may fuse without nuclear fusion; (ii) karyogamy, nuclear fusion; (iii) meiosis, creating specialized haploid spores; and (iv) growth of haploid mycelia. Table 5.4 provides a summary of the properties of the five major traditional groups of fungi. Their common habitats range from soil to water to decaying plant material. One crucial ecological role of fungi,

Figure 5.10 Photograph of fungal fruiting bodies on a forest tree in Ithaca, NY. (Courtesy of E.L. Madsen.)

mycorrhizae (literally "fungus root"), is thought to stem from coevolution with land plants. Mycorrhizal symbiotic relationships between soil fungi and root tissues lead to enhanced nutrient (especially phosphorus) uptake by the fungal symbiotic partner in exchange for plant photosynthate (provided by the plant partner; see Section 8.1).

For additional information on fungi, see Dugan (2006), Xu (2005), Spooner and Roberts (2005), Lundquist and Hamelin (2005), Dighton et al. (2005), Petersen et al. (2004), Hirt and Horner (2004), Dighton (2003), Burnett (2003), Kirk et al. (2001), Stephenson (2010), Barkovich and Ebbole (2010), and Hibbett and Taylor (2013).

Protozoa

Traditionally, protozoa have been defined to be unicellular forms of life whose structures and behavior set them apart from the two prokaryotic domains and from other branches in the *Eukarya* (especially fungi and algae; see Figure 5.7). The biology and taxonomy of traditional protozoology (based on phenotypic traits such as size, structure, and behavior) is revealed by the tree of life to span an enormous evolutionary distance – from "ancient" representatives residing close to the evolutionary emergence from prokaryotes to sophisticated, highly evolved ciliates. The range of structural, ecological, and nutritional protozoan traits is vast (see Table 5.5,

Table 5.4

Properties of the major traditional groups of fungi. (Modified from Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 469. Prentice Hall, Upper Saddle River, NJ. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ)

Traditional fungal group	Taxonomy based on Figure 5.8 phylogeny	Common name	Hyphae	Typical representative	Type of sexual spore	Habitats	Common diseases caused
Ascomycetes	Opisthokont (Fungi)	Sac fungi	Septate	*Neurospora*, *Saccharomyces*, *Morchella* (morels)	Ascospore	Soil, decaying plant material	Dutch elm, chestnut blight, ergot, rots
Basidiomycetes	Opisthokont (Fungi)	Club fungi, mushrooms	Septate	*Amanita* (poisonous mushroom), *Agaricus* (edible mushroom)	Basidiospore	Soil, decaying plant material	Black stem, wheat rust, corn smut
Zygomycetes	Opisthokont (Fungi)	Bread molds	Coenocytic	*Mucor*, *Rhizopus* (common bread mold)	Zygospore	Soil, decaying plant material	Food spoilage; rarely involved in parasitic disease
Oomycetes	Stramenopile (Oomycete)	Water molds	Coenocytic	*Allomyces*	Oospore	Aquatic	Potato blight, certain fish diseases
Deuteromycetes	NA	Fungi imperfecti	Septate	*Penicillium*, *Aspergillus*, *Candida*	None known	Soil, decaying plant material, surfaces of animals bodies	Plant wilt, infections of animals such as ringworm, athlete's foot, and other dermatomycoses, surface or systemic infections (*Candida*)

NA = Not applicable. The fungi imperfecti are defined by the absence of an observed sexually reproduction stage. This absence is not relevant to the taxonomic schemes in Figure 5.8.

Table 5.5

Properties of the major traditional groups of protozoa. (Modified from Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 464. Prentice Hall, Upper Saddle River, NJ. Copyright 2006, reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ)

Traditional grouping based on phenotype	Taxonomy based on Figure 5.8 phylogeny	Typical representative	Nutrition	Habitats	Common diseases caused
Mastigophore (flagellates)	Discicristates, Opisthokonta	*Trypanosoma*, *Leishmania*, *Trichomonas*	Osmotroph, phagotroph	Freshwater, soil; animal parasites	African sleeping sickness, leishmaniasis
Euglenoids (phototrophic flagellates)	Discicristates (Euglenid)	*Euglena*	Phototroph	Freshwater, soil, some marine	None known
Sarcodina (amoebae)	Amoebozoa,Rhizaria	*Amoeba*, *Entoamoeba*	Phagocystosis	Freshwater, soil, marine; animal parasites	Amoebic dysentery (amoebiasis)
Ciliophora (ciliates)	Alveolates	*Balantidium*, *Paramecium*, *Tetrahymena*	Ingestion through mouth-like opening	Freshwater, soil, marine; animal parasites; rumen	Dysentery
Apicomplexa (sporozoans)	Excavates	*Plasmodium*, *Toxoplasma*, *Cryptosporidium*	Osmotroph	Primarily animal parasites, insects (vectors for parasitic diseases)	Malaria, toxoplasmosis, cryptosporidiosis
Diplomonad (mitochondria absent)	Excavates	*Giardia*	Osmotroph	Freshwater; obligate animal parasites	Giardiasis
Microsporidia (mitochondria absent)	Opisthokonta	*Encephalitozoon*	Osmotroph	Freshwater; obligate animal parasites	Tissue infections (muscles, lungs, gastrointestinal tract)

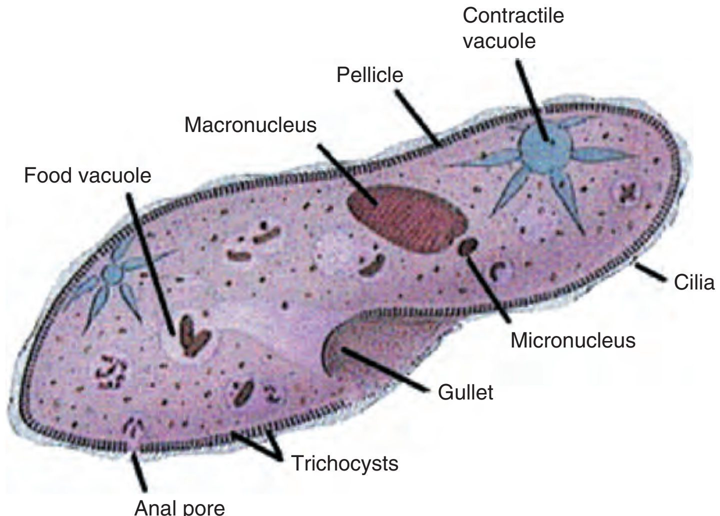


Figure 5.11 Diagram of the cellular architecture of the ciliate protozoan, *Paramecium*. (From Purves, W.K., G.H. Orians, and H.C. Heller. 1992. *Life: The science of biology*, 3rd edn. Sinauer Associates, Inc./W.H. Freeman and Co., Sunderland, MA. Reprinted with permission.)

Figure 5.8, and text accompanying Figure 5.8). Figure 5.11 shows a diagram of a ciliated protozoan. The one feature common to all protozoa is a membrane-bound nucleus – but other common assumptions about widespread eukaryotic features stop there. As mentioned above, some protozoa (e.g., diplomonads and microsporidia) lack mitochondria and other common organelles. Instead of mitochondria, these often anaerobic cells contain "hydrogenosomes" [mitochondria-like organelles that convert pyruvate to adenosine triphosphate (ATP), H_2, acetate, and CO_2] and/or a small, poorly understood, sack-like structure termed a mitosome. Phylogenetic analysis of hydrogenosome genes indicates that, like mitochondria and chloroplasts, hydrogenosomes were bacterial endosymbionts (related to the present-day bacterial genus *Clostridium*) engulfed by ancient anaerobic eukaryotic cells. Another feature of microsporidia and diplomonads is that they contain 16S, not 18S, rRNA! Both lack of mitochondria and the presence of 16S rRNA have traditionally been interpreted as being indicative of a "primitive", less-evolved state for diplomonads and microsporidia. However, as mentioned above, an alternative interpretation ("specialized evolution leading to loss of function", shown in Figure 5.8 and accompanying text) now prevails. In fact, recent molecular phylogenetic analyses using gene sequences other than small subunit rRNA have argued for taxonomic relocation of microsporidia in the tree of life to a position very close to the fungi (Figure 5.8).

Motility via flagella (whip-like structures) is thought to have been an early step in protozoan evolution. Flagella are a readily recognizable distinguishing phenotype – giving rise to the terms "flagellates" and "mastigophora" in protozoology (Table 5.5). However, phylogenetically, flagella are of little significance because the trait is widespread among the many protozoan taxa. From a developmental standpoint, it is reasonable to presume (Sleigh, 1989) that ancestral flagellated protozoa made the transition to both amoeboid motility (via loss of flagella) and to ciliated motility (via multiplication and diminution of flagella). Another key force in protozoan evolution was incorporation of the chloroplast organelle. In this regard, the *Euglena* line of descent was reasonably successful (see Figures 5.7 and Figure 5.8); these organisms are phototrophic and highly motile via flagella. This mixture of

animal-like motility and plant-like nutrition (photosynthesis) blurs the traditional taxonomic and disciplinary lines in protozoan biology. Another example of blurry taxonomy is the golden-brown alga, *Ochromonas*: this organism is flagellated and photosynthetic. Is *Ochromonas* a protozoan or an alga? Inability of traditional taxonomic procedures to address such questions reveals the critical role that molecular phylogeny plays in modern biology (see Figure 5.8).

Even the most highly evolved ciliates (Table 5.5) are a challenge to understand because of their unique cellular features. Many ciliates carry both a micronucleus (diploid, critical for inheritance and sexual reproduction, but transcriptionally inactive) and a macronucleus (derived from the micronucleus, polyploid, and the sole site of gene expression, via RNA transcription, for the cell). Another structural oddity of some protozoa is the "apical complex", a unique cluster of rings and microtubules involved in biosynthetic processes and attachment. The presence of the apical complex is the traditional defining trait for the Apicomplexa (sporozoan) group of protozoa (Table 5.5). Members of the Apicomplexa are obligate parasites of insects and animals that produce resistant sporozoite stages that facilitate the organism's survival during transmission from one animal host to the next.

The diverse examples of endosymbionts, nutritional constraints, and structural modification in protozoan biology are impressive, and so also are the ecological and medical impacts of protozoa (Table 5.5, Figure 5.8 and accompanying text). Colonies of protozoa (e.g., *Volvox* and marine protozoa known as radiolarians) can be relatively large in size (measureable in centimeters) and exhibit spatial specialization resembling tissues in higher *Eukarya*. As free-living bacterivores in soil and aquatic habitats, predatory protozoa often play important roles in microbial food chains. Grazing upon prokaryotic cells can enhance their growth rate and hence stimulate the biogeochemical processes that prokaryotes catalyze (Fenchel, 1987). Furthermore, the protozoan biomass is, itself, an important component of the trophic pyramid (see Section 7.4), as protozoa are fed upon by zooplankton (in aquatic habitats) and nematodes, mites, or insects (in soil). From a medical perspective, a plethora of important human and animal diseases (from dysentery to malaria) are caused by protozoa.

For additional information on protozoa, see Sterling and Adam (2004), Clarke (2003), Lee et al. (2000), Smith and Parsons (1996), Margulis et al. (1993), Sleigh (1989), Fenchel (1987), and Castillo and Harris (2013).

Slime molds (Amoebozoa)

18S rRNA analysis shows that slime molds are more ancient than fungi but more evolved than *Euglena* (see Figure 5.7). Using the more recent taxonomy, slime molds are eukaryotes closely related to amoeboid protozoa, falling within the Amoebozoa supergroup (Figure 5.8). Figure 5.12 shows

Figure 5.12 Photograph of slime mold fruiting bodies on forest foliage in Olympic National Park, Washington State, USA. (From Olympic National Park, with permission.)

a photograph representative of slime molds on forest foliage. The key trait of slime molds is that they exhibit colonial behavior in which large motile masses of amoebae move to engulf and feed on decaying plant material and bacteria in soil or on fallen trees. The colonial stage alternates with a morphologically specialized stage that produces spore-forming structures. After release, the spores germinate into haploid swarm cells. Compatible swarm cells fuse into diploid vegetative amoebae that grow, reproduce, and cycle back to the colonial stage.

Based on morphology, especially the presence or absence of cell walls, there are two types of slime molds: cellular and acellular. In the acellular slime molds (e.g., *Physarum*) the vegetative masses of indefinite size are termed "plasmodia", whereas in the cellular slime molds (e.g., *Dictyostelium*) the aggregated cells are termed "pseudoplasmodia". The cellular slime molds have been extensively studied at the biochemical and genetic level, as a model for understanding cellular differentiation. The ecological impact of slime molds is not well explored (e.g., Landolt et al., 2004). For additional information on slime molds see Margulis et al. (1993), Stephenson and Stempen (1994), Wilkinson et al. (2012), and Hoppe (2013).

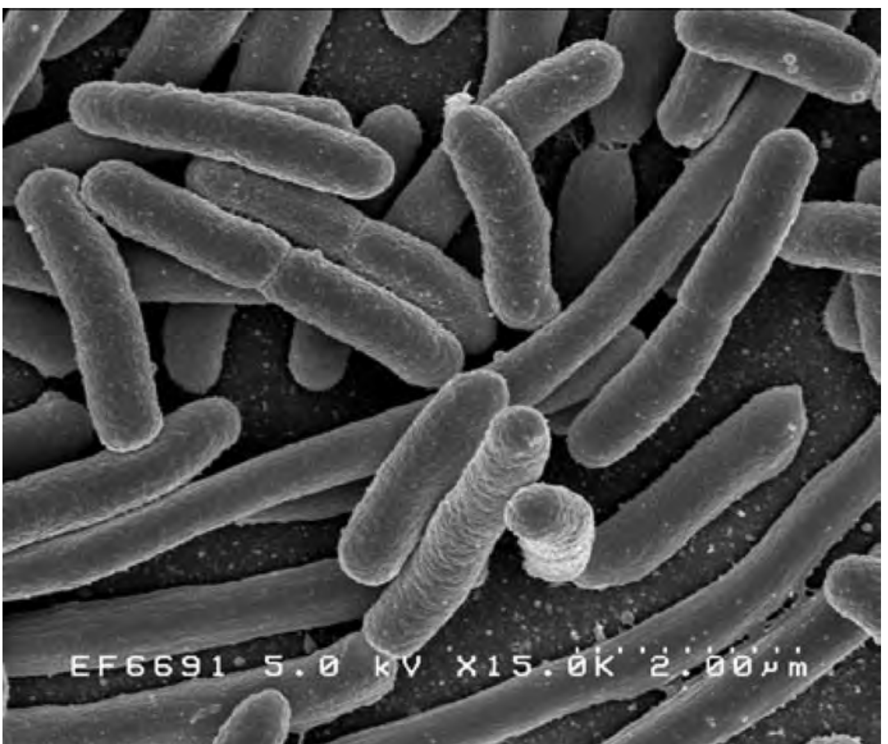


Figure 5.13 Scanning electron micrograph of the γ-Proteobacteria, *E. coli*, grown in culture and adhered to a glass surface. (From Rocky Mountain Laboratories, NIAID, NIH, with permission.)

The domain *Bacteria*

Bacteria are prokaryotes that are distinctive from *Archaea* (Figure 5.7). The critical distinction is in the 16S rRNA gene sequences. Figure 5.13 shows a photograph representative of the common γ-Proteobacteria, *Escherichia coli*. Additional phenotypic distinctions include: ester linkages in membrane lipids, muramic acid in cell walls, protein synthesis initiated by formylmethionine tRNA, a single type of four-subunit RNA polymerase, Pribnow box-type promoter structure for transcription of genes, sensitivity to certain protein-synthesis inhibitors (chloroamphenicol, streptomycin, kanamycin), and absence of growth above 100 °C; some members carry out chlorophyll-based photosynthesis (see Table 2.3). As mentioned in Section 5.1 and Table 5.1, the most recent version of *Bergey's Manual of Systematic Bacteriology* (Garrity et al., 2005) lists 24 phyla of *Bacteria*. Descriptions of all 24 phyla appear in the manual and are expanded upon at lower taxonomic levels in other related works (e.g., Brenner et al., 2005; Dworkin et al., 2006; Madigan et al., 2014; Bergey et al., 2012). A complete survey of all

24 phyla is beyond the scope of this chapter; however, descriptions based on Garrity et al. (2005) of the eight bacterial phyla shown in Figure 5.7 appear below. Distinctions between Gram-negative and Gram-positive microorganisms were presented in Box 2.3.

Aquifex

Aquifex/Hydrogenobacter is the deepest and earliest branching phylum of *Bacteria*. All members are Gram-negative, nonsporulating rods or filaments with optimum growth in the range of 65–85 °C. These are chemolithoautotrophs or chemolithoheterotrophs using H_2, S^0, or $S_2O_4^{2-}$ as electron donors and O_2 or NO_3^- as electron acceptors.

Thermotoga

Thermotoga is also an early, deeply branching phylum. All members are Gram-negative, nonsporulating, rod-shaped bacteria that possess a characteristic sheath-like outer layer or "toga". *Thermotoga* are strictly anaerobic heterotrophs, utilizing a broad range of organic compounds as carbon sources and electron donors. Thiosulfate and S^0 are electron acceptors.

Chloroflexi (green nonsulfur bacteria)

Chloroflexi, another deep-branching bacterial lineage, are Gram-negative, filamentous organisms exhibiting gliding motility. Members of this phylum are physiologically diverse. Some (*Chloroflexus*) contain bacteriochlorophyll and are obligate or facultative anoxygenic phototrophs, exhibiting what may be the earliest form of photosynthetic reaction centers and CO_2 fixation mechanisms (see Section 2.10). The electron donors for CO_2 reduction are H_2 and H_2S; sulfur granules do not accumulate. Other members of this phylum do not contain bacteriochlorophyll and are chemoheterotrophs.

Chlorobi (green sulfur bacteria)

Chlorobi are Gram-negative, spherical, ovoid, straight, curved, or rod-shaped cells that are strictly anaerobic and obligately phototrophic. Cells grow preferentially on fixed organic compounds in the light (photoheterotrophy). Some species may utilize sulfide or thiosulfate as electron donors for CO_2 fixation. Sulfur granules accumulate on the outside of the cells when grown on sulfide in the light, and sulfur is rarely oxidized further to sulfate. Ammonia and N_2 gas are used as nitrogen sources.

Bacteroidetes

Bacteroidetes share a common phylogenetic root with green sulfur bacteria, yet the branching depth within each group warrants the phylum category.

The phylum is split into three major sublineages, *Bacteroidetes*, *Flavobacteri*, and *Sphingobacteria*, which range from obligate anaerobes to obligate aerobes. The broad phenotypic characteristics of this group includes: Gram-negative aerobic or microaerophilic rods; Gram-negative anaerobic rods; nonphotosynthetic, nonfruiting, gliding bacteria; bacterial symbionts of invertebrate species; sheathed bacteria; and nonmotile or rarely motile, curved Gram-negative bacteria.

Cyanobacteria

Cyanobacteria are Gram-negative, unicellular, colonial, or filamentous oxygenic and photosynthetic bacteria exhibiting complex morphologies and life cycles. The principal characters that define all members of this phylum are the presence of two photosystems (PSI and PSII; see Section 2.10) and the use of H_2O as the reductant in photosynthesis. All members contain chlorophyll *a* with or without other light-harvesting pigments. Although facultative photoheterotrophy or chemoheterotrophy may occur in some species or strains, all known members are capable of photoautotrophy (using CO_2 as the primary source of cell carbon).

Proteobacteria

Proteobacteria (also known as purple bacteria) constitute the largest phylogenetic group within the *Bacteria*, divided into five major subdivisions (α, β, γ, δ, and ε), composed of more than 520 genera. Phenotypic groups within the Proteobacteria include: Gram-negative aerobic or microaerophilic rods and cocci; anaerobic straight, curved, and helical Gram-negative rods; anoxygenic phototrophic bacteria; nonphotosynthetic, nonfruiting, gliding bacteria; aerobic chemolithotrophic bacteria and associated genera; facultatively anaerobic Gram-negative rods; budding and/or appendaged nonphototrophic bacteria; symbiotic and parasitic bacteria of vertebrate and invertebrate species; and fruiting, gliding bacteria. As a group, these are all Gram-negative, show extreme metabolic diversity, and represent the majority of Gram-negative bacteria of medical, industrial, and agricultural significance.

Gram-positive bacteria

Gram positives are found in two phyla, the *Firmicutes* and *Actinobacteria*.

The *Firmicutes* constitute an extensive phylum of >1500 species within >220 genera spanning three main subdivisions: the *Clostridia*, the *Mollicutes*, and the *Bacilli*. This phylum, though dominated by Gram-positive microorganisms, is phenotypically diverse and includes some members with Gram-negative cell walls. Phenotypic groups of the *Firmicutes* include thermophilic bacteria; anaerobic, straight, curved, and helical Gram-negative rods; anoxygenic phototrophic bacteria; nonphotosynthetic, nonfruiting,

gliding bacteria; aerobic nonphototrophic chemolithotrophic bacteria; dissimilatory sulfate- or sulfite-reducing bacteria; symbiotic and parasitic bacteria of vertebrate and invertebrate species; anaerobic Gram-negative cocci; Gram-positive cocci; endospore-forming Gram-positive rods and cocci; regular and irregular nonsporulating Gram-positive rods; mycoplasmas; and thermoactinomycetes.

Actinobacteria are the second phylum of Gram-positive *Bacteria* – phylogenetically related to, but distinct from, the *Firmicutes*. *Actinobacteria* feature a high level of morphological, physiological, and genomic diversity that falls within this phylum's six major taxonomic orders and 14 suborders. The phylum can be broadly divided into two major phenotypic groups: unicellular, nonsporulating *Actinobacteria* and the filamentous, sporulating *Sporoactinomycetes*. The unicellular *Actinobacteria* include some Gram-negative aerobic rods and cocci; aerobic, sulfur oxidizing, budding and/or appendaged bacteria; Gram-positive cocci; regular nonsporulating, Gram-positive rods; irregular non-sporulating, Gram-positive rods; and mycobacteria. The *Sporoactinomycetes* include noncardioform actinomycetes, actinomycetes with multicellular sporangia, actinoplanetes, *Streptomyces* and related genera, maduromycetes, *Thermomonospora* and related genera, and other sporoactinomycete genera.

The domain *Archaea*

Archaea are prokaryotes that are distinctive from *Bacteria* (Boone and Castenholz, 2001). The critical distinction is in the 16S rRNA gene sequences. Figure 5.14 shows a photograph of *Methanosarcina*, a member of the *Archaea*. Additional phenotypic distinctions include: histone proteins associated with chromosomal DNA; absence of muramic acid in cell walls; ether-linked membrane lipids (see Section 2.7); protein synthesis initiated by methionine tRNA; several types of RNA polymerases (8–12 subunits each); TATA box-type promoter structure for transcription of genes; insensitivity to bacterial-type protein synthesis inhibitors; some members are methanogenic; no members carry out chlorophyll-based photosynthesis; and some members are able to grow at temperatures above 100 °C (see Section 2.12 and Table 2.3).

As mentioned in Section 5.1 and Table 5.1, there are five cultured phyla of the *Archaea* as shown in the tree of life: the *Crenarchaeota*, the *Euryarchaeota*, the *Thaumarchaeota*, the *Korachaeota*, and the *Nanoarchaeota* (see Figure 5.7). Brief descriptions of the five phyla and several of the pertinent taxonomic subdivisions shown in Figure 5.7 appear below (Brochier-Armanet et al., 2011; Pester et al., 2011; Spang et al., 2010, 2013).

Crenarchaeota

The *Crenarchaeota* consist of a single class, the *Thermoproteus*, which is composed of three orders: *Thermoproteales*, *Desulfurococcides*, and

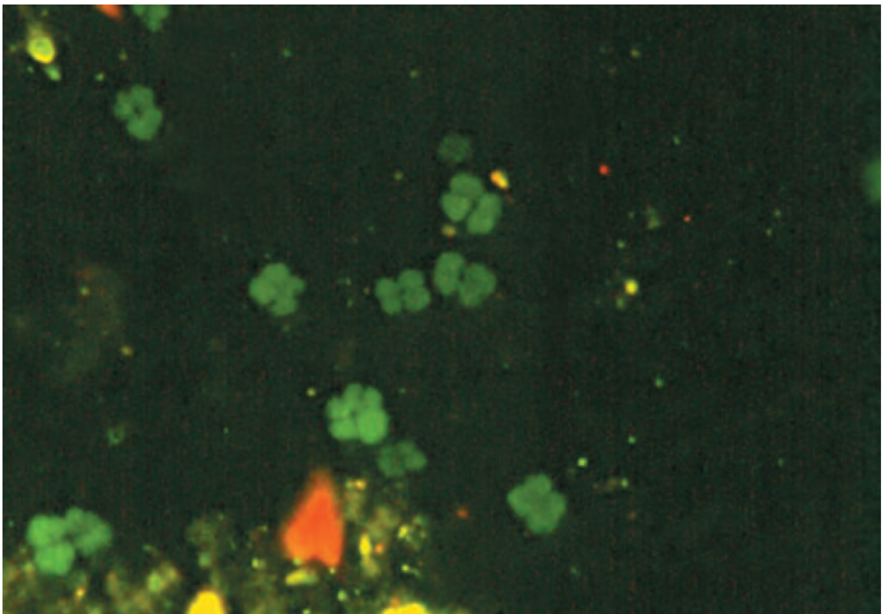


Figure 5.14 Epifluorescent photomicrograph of a representative of the *Archaea*, *Methanosarcina*, a methanogen. Note the clusters of four green fluorescent cells characteristic of this genus. (From S.H. Zinder, Cornell University, with permission.)

Sulfolobales. Members of the phylum are morphologically diverse, including rods, cocci, filamentous forms, and disk-shaped cells that stain Gram-negative. Motility is observed in some genera. The organisms are obligately thermophilic, with growth occurring at temperatures ranging from 70 to 122 °C. The organisms are acidophilic and are aerobic, facultatively anaerobic, or strictly anaerobic chemolithoautotrophs or chemoheterotrophs. Most metabolize S^0. Chemoheterotrophs may use organic compounds as electron donors and S^0 as an electron acceptor (reducing it to H_2S). Recent genomic surveys have indicated that a biomarker characteristic of the *Crenarchaeota* (and shared with *Korarchaeota*) is RNA polymerase subunit Rpb8 (Brochier-Armanet et al., 2011). Members of this phylum have been isolated from many of the most extreme environments on Earth – hot springs, sulfataras, hydrothermal vents, etc. For this reason the cultured *Crenarcheota* have provided a wealth of information about physiological and metabolic diversity.

Euryarchaeota

The Euryarchaeota is a phylum consisting of ten classes: *Methanobacteriales*, *Methanococales*, *Methanomicrobiales*, *Halobacteriales*, *Thermoplasmatales*, *Thermococcales*, *Archaeoglobales*, *Methanopyrales*, *Methanocellales*, and DHVE 2

(deep-sea hydrothermal vent 2). In phylogenetic analyses (e.g., Figure 5.7), many phyla share a common root; therefore, several phylogenetic relationships are ambiguous when only 16S rRNA genes are considered (Garrity et al., 2005). However, phylogenetic relationships have become clearer using sequences for 57 ribosomal proteins as criteria (Brochier-Armanet et al., 2011). The *Euryarchaeota* are morphologically diverse and occur as rods, cocci, irregular cocci, lancet shaped, spiral shaped, disk shaped, triangular, or square cells. Cells stain Gram-positive or Gram-negative based on the absence or presence of pseudopeptidoglycan (a peptidoglycan analog) in cell walls. In some cases, cell walls consist entirely of protein or may be entirely absent. At least six major physiological groups have been described: methanogenic *Archaea*, extremely acidophilic *Archaea*, extremely halophilic *Archaea*, *Archaea* lacking a cell wall, sulfate-reducing *Archaea*, and extremely thermophilic S^0 metabolizers.

Thaumarchaeota

The *Thaumarchaeota* phylum (Figure 5.7) was recognized only recently (Brochier-Armanet et al., 2011; Spang et al., 2010; Pester et al., 2011). The four contributing classes are: *Cenarchaeum*, *Nitrosopumilus*, *Nitrososphaera*, and *Candidatus* Caldiarchaeum. Initial clues for the existence of the *Thaumarchaeota* were obtained from early environmental surveys of 16S rRNA genes in ocean surface waters (DeLong et al., 1992; Fuhrman et al., 1992; DeLong, 1998). The widespread occurrence of archael genes related to hyperthermophilic *Crenarchaeota* in a cool marine habitat challenged existing views about the physiology and ecology of *Archaea*. Physiological characterization and/or complete genome sequencing of four far-ranging microorganisms contributed in a major way to establishing the *Thaumarchaeota*. The four microbes were: (i) the sponge endosymbiont, *Candidatas* Cenarchaeum symbiosum; (ii) the marine ammonia oxidizer, *Nitrosopumilus maritimus*; (iii) an inhabitant of the geothermal waters in a subsurface gold mine (*Candidatus* Caldiarchaeum subterraneum); and (iv) the terrestrial, soil-derived ammonia oxidizer, *Nitrososphaera gargensis*. This (and other) information was used to assemble a phylogenetic analysis (based on the sequences of 53 ribosomal proteins and FtsZ, CdvBC, and actin, among other proteins) to establish the new phylum. The *Thaumarchaeota* are globally distributed and their ammonia-oxidizing, autotrophic (CO_2-fixing) physiological traits ensure that the ecological significance of this phylum will be explored more fully in the future.

Korarchaeota

In 1996 the phylum *Korarchaeota* (Figure 5.7) was proposed based on 16S rRNA sequences retrieved from a hot spring in Yellowstone National Park (Barns et al., 1996). In 2008, after a decade of cultivation efforts, a

genomic sequence was obtained for the first representative organism of this phylum: *Candidatus* Korarchaeum cryptofilum is a hyperthermophilic (85 °C) heterotrophic anaerobe originating from Obsidian Pool, Yellowstone National Park, whose morphology shows long, ultrathin filaments. Many widely distributed biosynthetic genes were absent from the genome of *Ca.* Korarchaeum cryptofilum; this suggests that key nutrients are provided by this organism's neighbors in its native habitat, hot-spring microbial mats. No one yet knows the ecological or physiological significance of members of the *Korarchaeota*. It is certain, however, that lessons from this first isolation and genome sequence will accelerate production of new knowledge about these microorganisms.

Nanoarchaeota

The *Nanoarchaeota* (Figure 5.7) represent a novel lineage within the *Archaea* based on the distinctive secondary structure of 16S rRNA. Huber et al. (2003) discovered the only isolated representative of *Nanoarchaeota*, named *Nanoarchaeum equitans*, which occurs as very small cocci (0.35–0.5 μm) singly or in pairs. The cell volume is approximately 1% that of typical *E. coli* cells. The sequenced genome is also very small (0.49 Mbp; see Sections 3.2 and 5.9). *N. equitans* cannot yet be grown in pure culture. The bacterium can be cocultured with a member of the Crenarchaeota in the genus *Ignicoccus*. *N. equitans* was found attached to the outer surface of *Ignicoccus* cells that originated from hydrothermal sediment samples in Iceland. The host, *Ignicoccus hospitalis*, is a strict anaerobe, growing optimally at 90 °C: *N. equitans* may be a parasite or a symbiont. Clues from the genome sequence promise to help explain *N. equitans*' physiology. Giannone et al. (2014) have recently used both proteomic and transcriptomic analyses (see Section 6.10) to investigate cellular and molecular foundations for the relationship between *N. equitans* and its host, *Ignicoccus*.

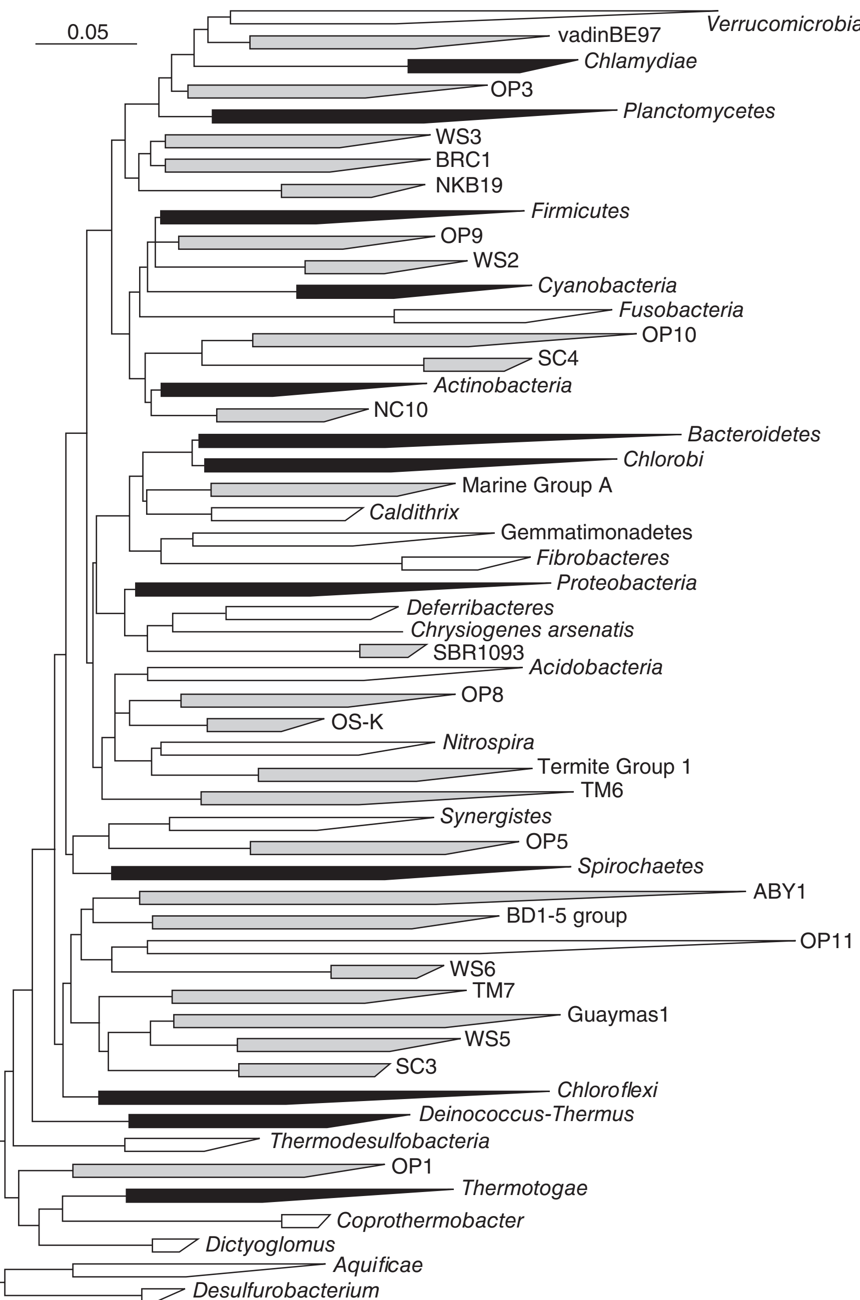
5.7 PLACING THE "UNCULTURED MAJORITY" ON THE TREE OF LIFE: WHAT HAVE NONCULTURE-BASED INVESTIGATIONS REVEALED?

Information presented in Chapter 4 (Section 4.5) provided estimates of the Earth's total prokaryotic biomass, as distributed across various habitats. Two related matters, how to operationally define and estimate microbial diversity, have been discussed in Sections 5.2 to 5.4. The current state of both eukaryotic and prokaryotic microbial taxonomy, based on cultured microorganisms, was presented in Sections 5.5 and 5.6. To strengthen the emerging portraits of the diversity of microorganisms, we now turn to information on uncultured microorganisms (defined in Section 5.1) using nonculture-based procedures.

An analysis of *Bacteria*

In 2003, Rappé and Giovannoni collected and analyzed accumulating 16S rRNA gene sequences directly derived from samples of freshwater, seawater, sediment, and soil. The authors' goal was to assess the impact of environmental libraries of 16S rRNA genes on our understanding of the diversity, evolution, and phylogeny of *Bacteria*. Figure 5.15 presents a picture of total bacterial phylogenetic diversity. The phylogenetic tree (Rappé and Giovannoni, 2003) integrates 16S rRNA sequence data from both cultured microorganisms (e.g., Figure 5.7) and noncultured microorganisms (Sections 5.1 and 5.2). The figure displays 52 phyla (major lineages) within the domain *Bacteria*. Each phylum is displayed as a "wedge" or "leaf" at the end of a branch. The size of the wedge representing each phylum reflects the number of accumulated sequences and the shading shows: (i) the 12 cultured phyla originally described by C. Woese in 1987 (black); (ii) the 14 additional phyla that were discovered through cultivation procedures between 1987 and 2003 (bringing the total to 26 (white)); and (iii) an additional 27 phyla discovered through nonculture-based sequencing of environmental 16S rRNA gene libraries (gray). The message from the information shown in Figure 5.15 is astounding. At a glance a reader can see that in one and a half decades (from 1987 to 2003), *phylum-level* assessment of bacterial diversity roughly doubled using cultivation-based techniques and *doubled again using 16S rRNA environmental gene-cloning procedures* devised by N. Pace and colleagues (Pace et al., 1986). The information portrayed in Figure 5.16

Figure 5.15 *(opposite)* Phylogenetic tree illustrating the major lineages (phyla) of the domain *Bacteria* (see text for details). Wedges shown in black represent the 12 original phyla, as described by Woese (1987); in white are the 14 phyla with cultivated representatives recognized since 1987; and in gray are the 26 candidate phyla that contain no known cultivated representatives. Horizontal wedge distances indicate the degree of divergence within a given phylum. The scale bar corresponds to 0.05 changes per nucleotide position. Phylum names are designated by selecting the first applicable option out of the following: (i) their convention in *Bergey's Manual of Systematic Bacteriology*, if it exists (Garrity et al., 2005); (ii) the first described representative genus within the phylum if it has cultivated representatives; (iii) the first label given to a candidate phylum if previously published; or (iv) the first clones or environment where the first clones were retrieved, for previously unnamed candidate phyla. This evolutionary distance dendogram was constructed by the comparative analysis of over 600 nearly full-length 16S ribosomal RNA gene sequences using the ARB sequence analysis software package, selected from a larger database of over 12,000 sequences. A modified version of the "Lane mask" was employed in this analysis, along with the Olsen evolutionary distance correction and neighbor-joining tree-building algorithm. (From Rappé, M.S. and S.J. Giovannoni. 2003. The uncultured microbial majority. *Annu. Rev. Microbiol*. **57**:369–394. Reprinted with permission from *Annual Reviews of Microbiology*, Vol. 57. Copyright 2003 by Annual Reviews, www.annualreviews.org.)



0.05
Verrucomicrobia
vadinBE97
Chlamydiae
OP3
Planctomycetes
WS3
BRC1
NKB19
Firmicutes
OP9
WS2
Cyanobacteria
Fusobacteria
OP10
SC4
Actinobacteria
NC10
Bacteroidetes
Chlorobi
Marine Group A
Caldithrix
Gemmatimonadetes
Fibrobacteres
Proteobacteria
Deferribacteres
Chrysiogenes arsenatis
SBR1093
Acidobacteria
OP8
OS-K
Nitrospira
Termite Group 1
TM6
Synergistes
OP5
Spirochaetes
ABY1
BD1-5 group
OP11
WS6
TM7
Guaymas1
WS5
SC3
Chloroflexi
Deinococcus-Thermus
Thermodesulfobacteria
OP1
Thermotogae
Coprothermobacter
Dictyoglomus
Aquificae
Desulfurobacterium

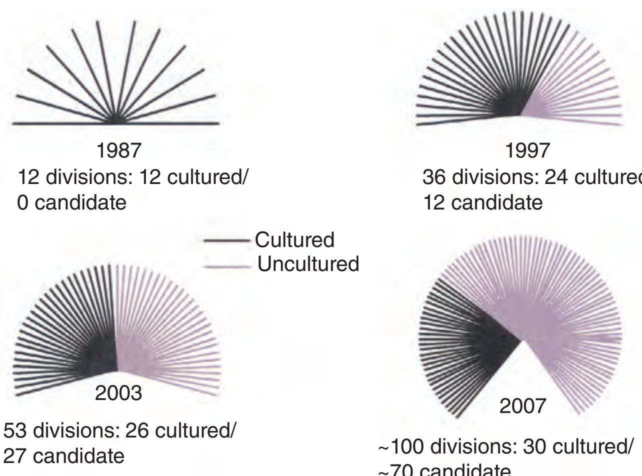


Figure 5.16 A view of bacterial 16S rRNA diversity over two decades. Shown are contrasting numbers of phyla ("divisions") represented by cultivated microorganisms (black) versus uncultured microorganisms (magenta). (From Sogin, M.L. 2009. Characterizing microbial populations structures through massively parallel sequencing. In: S.S. Epstein (ed.), *Uncultivated Microorganisms*, pp. 19–33. Springer-Verlag, Berlin, Heidelberg. With permission from Springer-Verlag, Berlin, Heidelberg.)

recapitulates and extends (to 2007) the pattern of discovery shown in Figure 5.15. Four years later, the total number of bacterial phyla ("divisions" in the figure) grew to ~100 and 70% of these have no cultivated representatives (Sogin, 2009).

"Microbial dark matter" is the phrase that has arisen to conceptually refer to the ubiquitous, mysterious, uncultivated (and hence extremely poorly characterized) component of microbial life (Marcy et al., 2007; Rinke et al., 2013). As mentioned in Sections 3.2 and 5.2, both single-cell genomics (see Section 6.9) and draft-genome assembly from metagenomic data (see Section 6.10) have bypassed the cultivation step as a prerequisite for acquiring genomic information. Recent efforts by the US Department of Energy's Joint Genome Institute have explicitly been focused on expanding genomic information about the "microbial dark matter" (Rinke et al., 2013). A 2014 review by Hedlund et al. (2014) indicates significant progress. The 26 bacterial phyla defined by cultivated *Bacteria* (see Section 5.1) have been expanded to 44 genomically defined phyla. These 44 currently known bacterial phyla are shown in Figure 5.17.

An analysis of *Archaea*

Environmental clone libraries of 16S rRNA genes have also significantly advanced knowledge of archaeal phylogenetic diversity. One way to appreciate such contributions is to refer to Figure 5.7, depicting five archaeal phyla: *Crenarchaeota*, *Euryarchaeota*, *Korarchaeota*, *Thaumarchaeota*, and *Nanoarchaeota*. Two of these phyla (*Crenarchaeota* and *Euryarchaeota*) have had long-standing verification from cultivated microorganisms. The *Nanoarchaeota* have but one cultivated representative (see Section 5.6), though biomarkers of this phylum are routinely retrieved from high-temperature marine and hypersaline habitats. By contrast, for many years clues about the existence of both the *Korarchaeota* and the *Thaumarchaeota* were provided only through preparation of clone libraries of 16S rRNA genes derived directly from environmental samples. Until very recently, cultivated members of the *Archaea* were only associated with

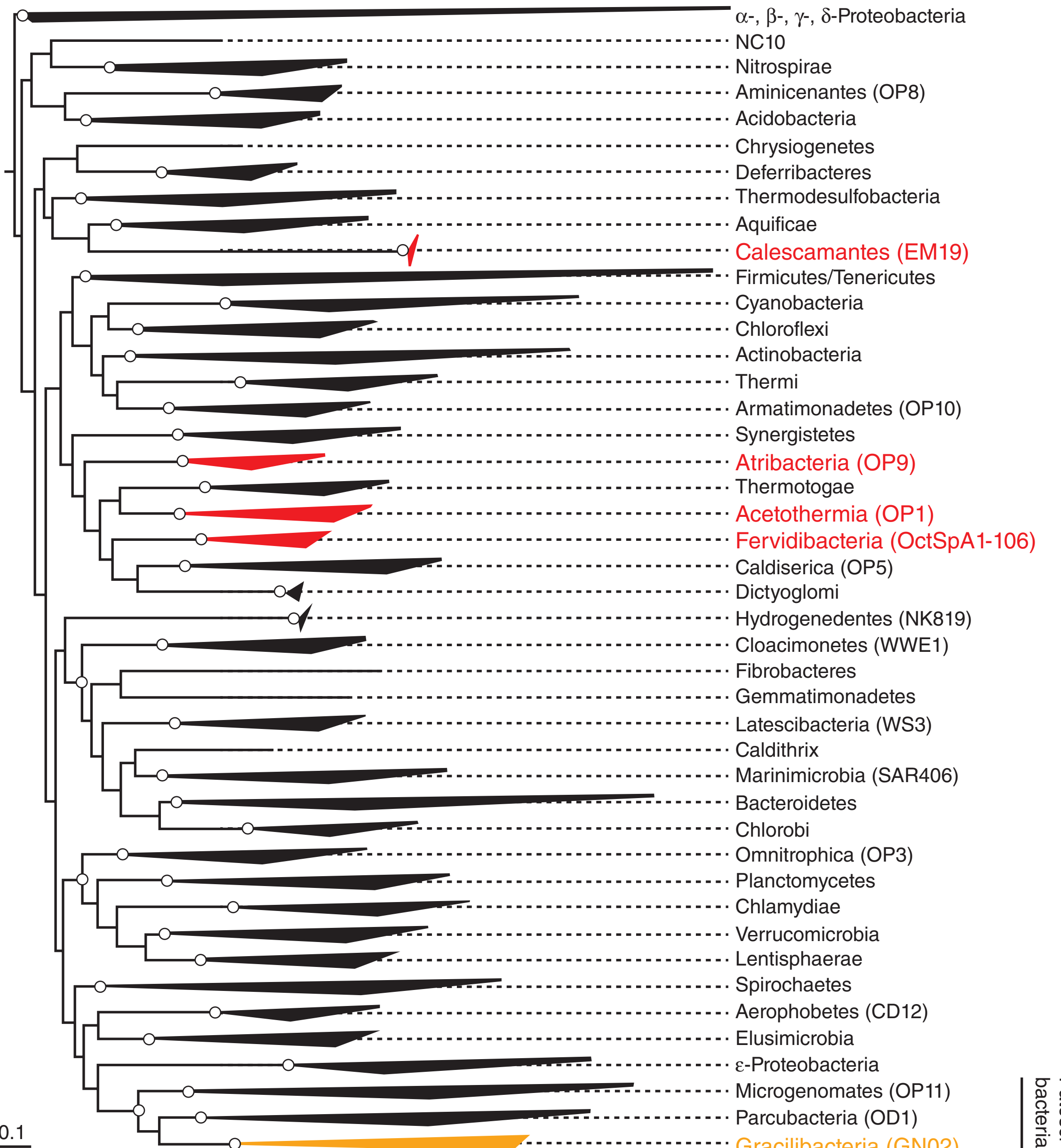


Figure 5.17 Maximum-likelihood consensus tree of *Bacteria* highlighting yet-uncultivated candidate phyla with complete or near-complete composite genomes determined by metagenomics or single-cell genomics. Tree is based on alignments of up to 38 conserved single-copy marker genes, modified from Rinke et al. 2013 (white circles, >90 % consensus). Colors highlight extremophiles and their habitats: red, thermophiles/terrestrial geothermal springs; yellow, piezophiles/piezotolerant/hydrothermal vents. Patescibacteria is a recently proposed superphylum recognizing shared lineage of Microgenomates, Parcubacteria, and Gracilibacteria. (Source: Hedlund, B.P., J.A. Dodsworth, S.K. Murugapiran, C. Rinke, and T. Woyke. 2014. Impact of single-cell genomics and metagenomics on the emerging view of extremophile “microbial dark matter”. *Extremophiles* **18**:865–875. Reprinted with permission of Springer Science + Business Media.)

"extreme" (i.e., thermophilic) phenotypes. However, as archaeal 16S rRNA gene sequences were increasingly discovered in habitats exhibiting moderate environmental conditions (such as soil, lake sediments, anaerobic digesters, tissues of marine animals, and cold, low-nutrient, marine waters and sediments; DeLong, 1998), new hypotheses were formulated about the ecological niches of *Archaea*.

Detection of the 16S rRNA sequences in unexpected habitats raised new questions about the potential impact and ecological roles of uncultivated Archaea. The answer to one such prominent question began to unfold when Könneke et al. (2005) reported cultivation and isolation of an autotrophic ammonia-oxidizing member of *Archaea*. As mentioned above in the description of cultivated *Thaumarchaeota*, *Nitrosopumilus* was a representative of mysterious ocean-dwelling cells whose 16S rRNA sequences had been discovered by Fuhrman and by DeLong a dozen years earlier. Clues that *Archaea* can carry out ammonia oxidation (a key step in the nitrogen cycle) were initially obtained from 16S rRNA clone libraries derived from laboratory-incubated ammonia-oxidizing estuary sediments and marine aquaria. After repeated transfers on enrichment media containing ammonia as an electron donor, oxygen as an electron acceptor, and bicarbonate as a carbon source, Könneke et al. (2005) isolated *Nitrosopumilus maritimus*, the first member of *Archaea* known to carry out ammonia oxidation. Because this new microorganism has now been characterized biochemically (Martens-Habbena et al., 2009) and genomically (Walker et al., 2010), *Nitrosopulimos maritimus* provides a window into the possible biogeochemical role of marine *Archaea* in cycling both carbon and nitrogen (see Sections 7.3 to 7.6). Particularly, germane to the influence of nonculture-based studies on knowledge of microbial diversity, early foundations of the entirely new archael phylum, *Thaumarchaeota* (with *Nitrosopumilus* as a key lineage), were established by nonculture-based environmental surveys of 16S rRNA genes.

A comprehensive examination of archaeal 16S rRNA sequences appeared in 2005 (Robertson et al., 2005). This revealed vast uncultured diversity of the *Archaea*. Robertson et al. (2005) contrasted cultured versus uncultured sources of 16S rRNA sequences (Figure 5.18). The computational algorithm used to assess phylogenetic relationships among 712 archael rRNA gene sequences shown in Figure 5.18 did not produce the current five-phylum view of *Archaea* (Figure 5.7). In the taxonomic depiction shown in Figure 5.18, the archaeal phylogeny features two main branches (phyla): the familiar *Crenarchaeota* and the *Euryarchaeota* (here *Crenarchaeota* absorb the *Korarchaeota*, *Nanoarchaeota*, and sequences that now comprise the *Thaumarchaeota*). Within each of the two main phyla shown in Figure 5.18, many evenly spaced taxonomic divisions emanate from a single branch. This radiating branching pattern is known as "polytomy" (Robertson et al., 2005). Regardless of the forms of the trees in Figure 5.18, the contrasts between cultivated and noncultured sources of sequences delivers the same astounding message for *Archaea* as was delivered for *Bacteria* in

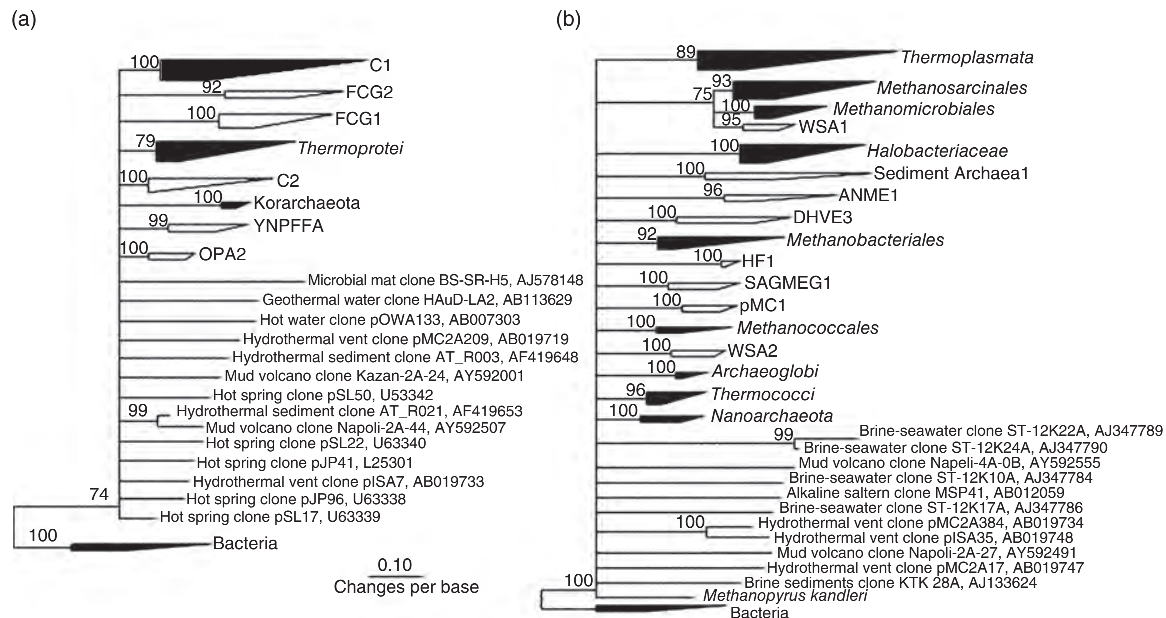


Figure 5.18 Phylogenetic tree illustrating the two major lineages (phyla) of the domain *Archaea* that were recognized in 2005: (a) Crenarchaeota and (b) Euryarchaeota. Solid colored groups have at least one cultured representative; others are known only from environmental samples. The sequences were downloaded from GenBank in February 2005 and were manually aligned using the ARB software. Three bacterial sequences were used as outgroups. PHYLIP software was used to generate 100 bootstrap data sets and to build the consensus tree that resulted from running the 100 data sets through RAxML. Any nodes in the tree that had less than 70% bootstrap support were deleted. ARB and PHYLIP are commonly used phylogenetic software tools. Phylogenetic relationships were generated with the RAxML software from 712 archaeal rRNA sequences that were at least 1250 nucleotides long. (Reprinted and modified from Robertson, C.E., J.K. Harris, J.R. Spear, and N.R. Pace. 2005. Phylogenetic diversity and ecology of environmental *Archaea*. *Curr. Opin. Microbiol.* **8**:638–642. Copyright 2005, with permission from Elsevier.)

Figures 5.15, 5.16, and 5.17. Taxa on the trees represented by solid symbols have cultivated representatives, while open symbols are from uncultured environmental sequences. Thus, within phylogenetic lines of descent shown as the *Crenarchaeota*, 18 of the 21 are not represented in culture. Within the phylogenetic lines of descent shown as *Euryarchaeota*, the proportion of uncultured lines amounts to 8 of 25.

Reviews of archaeal diversity that have appeared since 2005, include those by Pace (2009), Auguet et al. (2010), and Spang et al. (2013). Evidence for yet-to-be-discovered *Archaea* continues to be amassed: according to Auguet et al. (2010), success in cultivation efforts has led to 54 archaeal species spanning 18 lineages, yet up to 49 archaeal lineages (lines of descent) are largely without cultivated representatives. In addition, Auguet et al. (2010) analyzed ~2000 archaeal 16S rRNA gene sequences

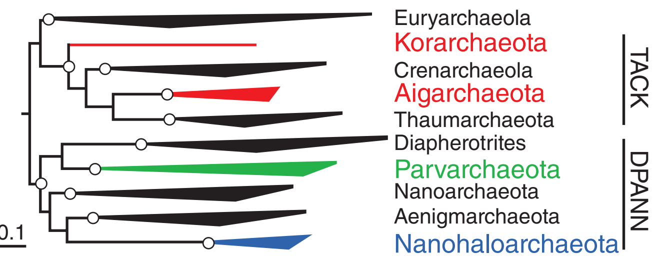


Figure 5.19 Maximum-likelihood consensus trees of *Archaea* highlighting yet-uncultivated candidate phyla with complete or near-complete composite genomes determined by metagenomics or single-cell genomics. Tree is based on alignments of up to 38 conserved single-copy marker genes, modified from Rinke et al. (2013) (white circles, >90 % consensus). Colors highlight extremophiles and their habitats: green, acidophiles/acid mine drainage; blue, halophiles/hypersaline lake; red, thermophiles/terrestrial geothermal springs. TACK, recently proposed superphylum recognizing shared lineage of Thaum-, Aig-, Cren-, and Kor-archaeota; DPANN, recently proposed superphylum recognizing shared lineage of Diaphertrites, Parv-, Aenigma-, Nano-, and Nanohalo-archaeota. (Source: Hedlund, B.P., J.A. Dodsworth, S.K. Murugapiran, C. Rinke, and T. Woyke. 2014. Impact of single-cell genomics and metagenomics on the emerging view of extremophile "microbial dark matter". *Extremophiles* **18**:865–875. Reprinted with permission of Springer Science + Business Media.)

from 67 globally distributed habitats (ranging from hot springs to soil to freshwater to ocean water to sediments). Hydrothermal vents and fresh waters emerged as the largest reservoirs of archael diversity; consequently, these habitats were deemed the most promising for discovery of new archaeal lineages.

As described for *Bacteria* above, expansion beyond 16S rRNA genes to *genome*-based inquiry into "microbial dark matter" (Marcy et al., 2007; Rinke et al., 2013) is a major scientific frontier. Like *Bacteria*, *Archaea* have been characterized by both single-cell genomics (see Section 6.9) and genome assembly from metagenomic data (see Section 6.10). In this way, draft genomes of uncultivated *Archaea* have been obtained directly from microbial communities residing in a variety of habitats. The goal of these investigations has been to expand genomic information about the archaeal component of "microbial dark matter" (Rinke et al., 2013). The 2014 review by Hedlund et al. (2014) on this topic indicates significant progress. The 5 phyla defined by cultivated *Archaea* (see Section 5.1) have been expanded to 10 genomically defined phyla. These 10 currently known archaeal phyla are shown in Figure 5.19.

An analysis of single-celled *Eukarya*

Section 5.6 presented a broad overview of current diversity among cultivated *Eukarya* (fungi plus protists). According to Caron et al. (2012), *Eukarya* fall into eight super groups (or phyla) that include: *Opisthokonts*, *Amoebozoa*, *Archaeplastida*, *Rhizaria*, *Alveolates*, *Stramenopiles*, *Discicristates*, and *Excavates* (Figure 5.8). Though generally much larger in size than prokaryotes (for exceptions see Section 5.9) and offering directly discernable morphological traits (size, motility, organelles, etc.), there are many parallels between protistan diversity and the diversity of *Bacteria* and *Archaea*. For example, there is no general agreement on how to define "species" in protists and there is likely a vast and rich,

yet-to-be-discovered diversity of protists in soils, waters, and sediments (Pawlowski et al., 2012).

- **What have nonculture-based surveys of 18S rRNA genes revealed about the true diversity of single-celled eukaryotes?**

One of the earliest forays into extraction and analysis of eukaryotic DNA sequences was completed by Dawson and Pace (2002). These researchers sampled three anoxic sediments (2 marine, 1 freshwater) and then extracted DNA and used the polymerase chain reaction (PCR) to amplify 18S rRNA genes. Next, they prepared clone libraries from the amplified genes (a total of >3000 clones) and obtained 125 unique eukaryotic small-subunit 18S rRNA gene sequences. After comparing these environmental gene sequences to the existing data set (~5000) from known organisms, Dawson and Pace (2002) concluded that they had discovered eight novel major lineages (kingdom-level evolutionary lines of descent). This new information expanded the *Eukarya* from 14 to 22 lineages. When Dawson and Pace's (2002) (and other researchers') work was re-examined two years later by Berney et al. (2004), several factors were identified that exaggerated the validity and novelty of some of the conclusions drawn by the pioneering environmental 18S rRNA gene surveys. After re-analysis of the previously published data, Berney et al. (2004) concluded that a total of five novel kingdom-level eukaryotic lines of descent had been unveiled through nonculture-based studies. Even with Berney et al's (2004) adjustments, the major finding remains: there is an immense amount of fundamental information about the diversity of single-celled eukaryotes yet to be discovered. Tackling this issue quantitatively, Pawlowski et al. (2012) have tallied the known number of cultivated protistan species to be ~60,000 – falling across the major groups (from Stramenopiles to Alveolates to Amoebozoa to Excavates). Yet Pawlowski et al. (2012) place the true richness of protistant species to be approximately 100-fold higher!

5.8 VIRUSES: AN OVERVIEW OF BIOLOGY, ECOLOGY, AND DIVERSITY

Viruses are one of the five classic forms of microbiological life (the others are prokaryotes (*Bacteria* plus *Archaea*), fungi, algae, and protists). Viruses, like prokaryotes, have domesticated representatives, upon which virtually all of the science of virology is based. Also, as for the other microorganisms (see Section 5.7), molecular environmental surveys have been performed to reveal a broad diversity of previously unknown viruses

(e.g., Breitbart, 2012; Steward et al., 2012; Hewson et al., 2010; Mokili et al., 2012). However, because viruses are obligate intracellular parasites (that lack protein synthesis capabilities), small subunit rRNA genes, essential for ribosomes, do not occur in viruses. Thus, there is no branch for viruses on the tree of life shown in Figure 5.7. However, molecular phylogenetic approaches have been recently applied to viruses (Hurst, 2000; Rohwer and Edwards, 2002; Mayo et al., 2005; Koonin et al., 2006; Lauber and Gorbalenya, 2012). Sakowski et al. (2014) have argued that genes encoding ribonucleotide reductases hold promise for revealing a universal virus phylogensy. Below is a brief summary of virus biology, ecology, and diversity.

Viruses are acellular agents of heredity and disease. They have two complementary definitions:

1. Genetic elements containing DNA or RNA that replicate in cells but have an extracellular state.
2. Obligate intracellular parasites whose DNA or RNA is encapsidated in proteins encoded by the viral genomes; the parasites have evolutionary histories independent of their hosts.

Viruses are particles in the size range of ~100 nm. A complete virus particle is termed a virion and is composed of the genetic nucleic acid core (genome) surrounded by a capsid shell formed from virus-encoded proteins. Viruses that infect bacteria are termed bacteriophages. Figure 5.20 shows an electron micrograph of a virus particle (Figure 5.20b) and the zones of clearing caused by a virus infecting a uniform layer (lawn) of its host bacterium (Figure 5.20a). The genome (DNA or RNA), housed in the

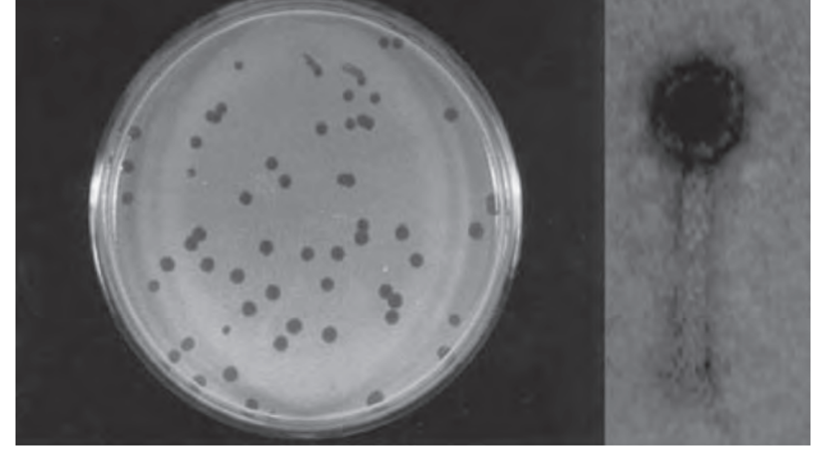


Figure 5.20 (a) Photograph of zones of clearing caused by viral infections of bacteria growing on an agar plate and (b) an electron micrograph of the virus revealing the head and tail structures. (From T. Nakai, Hiroshima University, with permission.)

capsid, is injected into the host bacterium after specific molecular recognition between components of the viral capsid and lipopolysaccharide in the outer membrane of the bacterium.

There are no traces or fossils of viruses in the geologic record. Preserved historical samples of virus-bearing tissue date back only to the early 1900s. Therefore, clues to the evolutionary history of viruses are limited to molecular phylogeny and educated speculation. There are at least four provocative (and untested) theories about the origin of viruses.

1. *Cell degeneration theory*. Independently functioning cells may have colonized a host, become intracellular, completely dependent upon host, and then gradually lost genetic traits.
2. *"Rebel" DNA or RNA*. Normal cellular components (DNA, RNA) may have gained the ability to replicate autonomously, and hence evolve; replicating nucleic acids in this family include viroids, virusoids, plasmids, and transposons.
3. *Ancient virus world*. Favored by Koonin et al. (2006), contemporary viruses may be remnants of ancient, self-replicating RNA precursors to modern life; these may have preceded cellular life and become intracellular parasites after *Bacteria* and *Archaea* evolved.
4. *Ancient parasites of the RNA world*. In this scenario, RNAs (the proto-viral genome) may have been enveloped by membrane vesicles. Then these "proto-virions" may have fused with and commandeered more sophisticated forms of cellular life. Later, protein-based capsids could have replaced the original membrane enclosure of the virions.

Viruses are sophisticated, highly evolved entities and their evolution continues today. As intracellular parasites, they generally have limitations on their size and shape. For RNA viruses (see below), there may also be limitations on genome size and structure imposed by the frequency of error during cellular RNA replication.

The genomes of today's viruses generally share three categories of gene clusters (Forterre and Prangishvili, 2009): (i) genes encoding the architecture of the capsid (which, together with the nucleic acid genomic core, forms the complete virion); (ii) genes encoding proteins that carry out genome replication; and (iii) genes that manipulate host function – converting an infected cell into what essentially becomes a factory for virion production (see below). There is no single line of descent for mapping the evolution of all viruses. Instead, viral taxonomists often portray virus evolution as a network of connected lineages – especially based on the sequences of genes encoding the highly conserved functions of capsid formation and genome replication (Forterre and Prangishvili, 2009).

Key structural aspects of virus biology include: their hereditary material (DNA or RNA, or DNA and RNA, as genetic material, at different life cycle stages); whether or not the nucleic acids are single stranded or double stranded, linear, or circular; capsid architecture, size, and shape; and the presence and type of lipid envelope that may surround the capsid. Medical aspects of viruses include characteristics such as disease type, host type (animals, plants, prokaryotes, protists), means of transmission, life cycle,

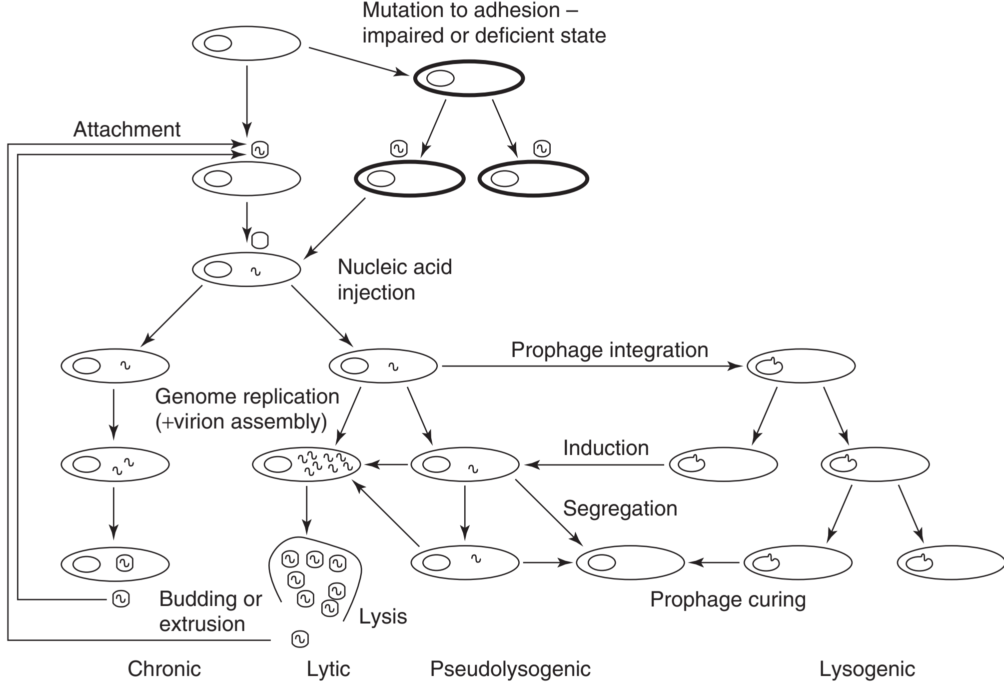


Figure 5.21 Types of viral life cycles. The model shows a typical bacterial cell with a circular chromosome (top), attachment by a virus or impaired attachment, and various stages of infection and intracellular viral behavior. See text for details. (From Weinbauer, M.G. 2004. Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* **28**:127–181. With permission from Blackwell Publishing, Oxford, UK.)

and infection specificity. Molecular mechanisms underlying the details of all aspects of viral biology, biochemistry, and replication deepen our understanding of life processes and may lead to treatment strategies that prevent infection (e.g., medicines) and cure diseases (e.g., gene therapy).

Figure 5.21 shows a typical life cycle for bacterial viruses and introduces the concept of lysogeny. Viruses can exhibit several types of life cycles: chronic, lytic, lysogenic, and pseudolysogenic. The chronic and lytic cycles are diagrammed on the left-hand side of Figure 5.21. They both proceed in five key steps in which the virus enters the cell and redirects host metabolism to create new virus particles released after host cell lysis. The five steps are: (i) attachment of the virion to a susceptible host cell; (ii) penetration (injection of the virion or its nucleic acid into the cell); (iii) alteration of the cell biosynthetic apparatus so that virally encoded enzymes and nucleic acids are generated; (iv) assembly of capsid shells and packaging of nucleic acids within them; and (v) release of mature virions from the cell. Note that, if attachment does not occur (Figure 5.21, upper center), infection is thwarted. As shown in Figure 5.21, the chronic and lytic cycles differ in the extent of intracellular virion assembly and the mode of release (nondestructive budding versus lysis and death of the host cell, respectively). In the lysogenic cycle (Figure 5.21, right), the viral genome integrates into

the host chromosome and replicates along with it, as if it were a normal component of the host chromosome. The genetically integrated phage DNA is known as a "prophage" because it resides in the host as a dormant precursor to an excised, potentially lytic, phage. When a suitable set of cellular triggers (a molecular dialog between the environment, host cell, and prophage) is activated, the prophage excises from the host chromosome and has the potential to begin the lytic cycle. Pseudolysogeny is the term applied to an intermediary state (between lysogenic and lytic stages) in which an extrachromosomal virus replicates in synchrony with the host chromosome (much like a plasmid).

The ecology of viruses and their impact on biogeochemical processes are active areas of research in environmental microbiology (e.g., Breitbart, 2012; Fuhrman, 1999; Wommack and Colwell, 2000; Weinbauer, 2004; Suttle, 2005; Hewson et al., 2010; Rohwer and Thurber, 2009; Matteson et al., 2010). Microscopic and isolation-based surveys from environmental samples have found that viral abundances exceed prokaryotic abundances and that a significant fraction of naturally occurring prokaryotic communities are infected by viruses. Figure 5.22 shows a seawater sample and its

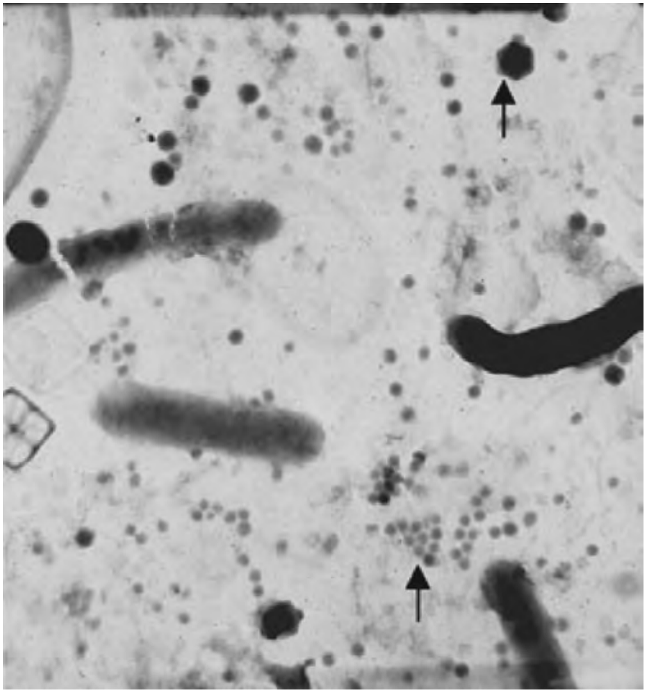


Figure 5.22 Transmission electron micrographs showing viruses and bacteria in a water sample from the northern Adriatic Sea. Viruses and bacteria were stained with uranyl acetate. Arrows point to viruses of different size. The large virus in the upper right corner has a head diameter of 150 nm. (From Weinbauer, M.G. 2004. Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* **28**:127–181. With permission from Blackwell Publishing, Oxford, UK.)

resident viruses. The abundance of bacterial viruses in soils, sediments, freshwaters, and ocean waters varies greatly and is related to host abundance and activity (Weinbauer, 2004). Recent estimates of abundances of viruses in seawater (from 10^5/ml in deep waters to 10^8/ml in productive coastal waters) have been extrapolated to yield several rather astonishing observations (Suttle, 2005; Hewson et al., 2010):

1. Freshwaters and marine waters support similar ranges of virus-like particles (10^5 to 10^8) per ml, with the lowest abundances in extremely low nutrient Antarctic lakes and the highest in freshwater portions of estuaries.
2. Globally, ocean waters may contain a total of ~4×10^{30} viruses (compare this to prokaryote numbers in the oceans; see Section 4.5).
3. Assuming that each virus contains 0.22 kg of carbon, viral biomass in the oceans amounts to 200×10^9 kg of carbon, second only to the biomass of prokaryotes (see Section 4.5).
4. Assuming an average length of 100 nm for each virus, if aligned end to end they would span a distance of 10 million light years.
5. The lytic cycle (see Figure 5.21) is the means by which viruses proliferate and evolve. Each lytic event ("burst" from a host cell) releases 24–34 progeny viruses in marine and freshwaters. Cycles of infection by viruses in marine habitats can lead to a rapid turnover of bacterial cells (e.g., Payet et al., 2014).
6. Ecologic success requires that at least one progeny virus from each burst contacts and reinfects a new host cell.
7. Decay of viruses in aquatic habitats stems from: enzymatic decomposition of viral nucleic acids, engulfment/consumption by small heterotrophic protozoa (i.e., nanoflagellates), damage by irradiation (especially UV light), and adsorption to particles, followed by sinking.
8. The lysogenic life cycle (see Figure 5.21) is considered favorable for virus survival when host cell densities are low and when host metabolic rates are low. Thus, the chromosomes of host cells serve as a refuge for viruses during hard times.
9. Genes carried by the infecting virus genome have been found to both: (i) contribute to host survival by repressing metabolic activity during lysogeny and (ii) encode proteins involved in a variety of key physiological traits (such as biosynthesis of nucleotides, uptake of phosphorus, photosynthesis, and other carbon processing) that boost host metabolism in ways that promote viral replication during the lytic cycle.

The terms "viriome" and "virome" describe the genetic core of a single virus. It follows that the terms "viral metagenomics" and "metaviriomics" apply to recent non-culture-based efforts that isolate virus particles directly from environmental samples and then use DNA sequencing technology (with or without clone preparation; see Sections 6.7, 6.9, and 6.10) to characterize naturally occurring viral communities. Such investigations have examined habitats ranging from human feces, to ocean water, to sampled marine sediments (Edwards and Rohwer, 2005; Hewson et al., 2010). Results have provided evidence for ~1000 viral genotypes in human

feces, ~5000 viral genotypes in 200 l of seawater, and up to 10^6 viral genotypes in marine sediment samples. The vast majority of these environmental virus sequences differ immensely from those of domesticated viruses.

As suggested above, lytic viruses threaten to obliterate prokaryotic populations, whereas lysogenic and chronic infections represent parasitic interactions. Clearly, virus-induced mortality of prokaryotes is highly variable in time and space – dependent especially on habitat characteristics, host abundances, and the host's physiological state (Payet et al., 2014). Because viral infections are host-specific and density-dependent, it is widely accepted that infections can control the composition of prokaryotic communities. Competitively dominant prokaryotes can be kept in check because viruses "kill the winner" (Weinbauer, 2004; Hewson et al., 2010; Breitbart, 2012). Regarding biogeochemistry, viruses are influential via at least three mechanisms: (i) their genomes can encode physiological traits (such as sulfur oxidation and phosphorus uptake; Anantharaman et al., 2014; Kelly et al., 2013) that augment ecological success of both the host cell and its parasitic virus; (ii) lytic cycles can reduce the sizes, and hence physiological activity of prokaryotes that catalyze individual steps in the cycling of carbon, nitrogen, sulfur, and other elements; and (iii) via the "viral shunt", nutrients can be moved from the cell-associated nutrient pool to the particulate and dissolved nutrient pool. Figure 5.23 illustrates the viral shunt for an oceanic habitat. In the classic view of trophic dynamics, microbial phytoplankton are grazed by zooplankton, which serve as food for carnivores. If lytic viruses divert phytoplankton biomass into the pool of dissolved and particulate organic matter, there are two potential consequences: the organic matter is utilized as a carbon source by heterotrophic bacteria (producing CO_2) and the flux of particular carbon (detritus) reaching and stored in marine sediments is diminished (Suttle, 2005). Both of these consequences may contribute to global warming (see Section 7.2).

According to Koonin et al. (2006), the paradigm for virus

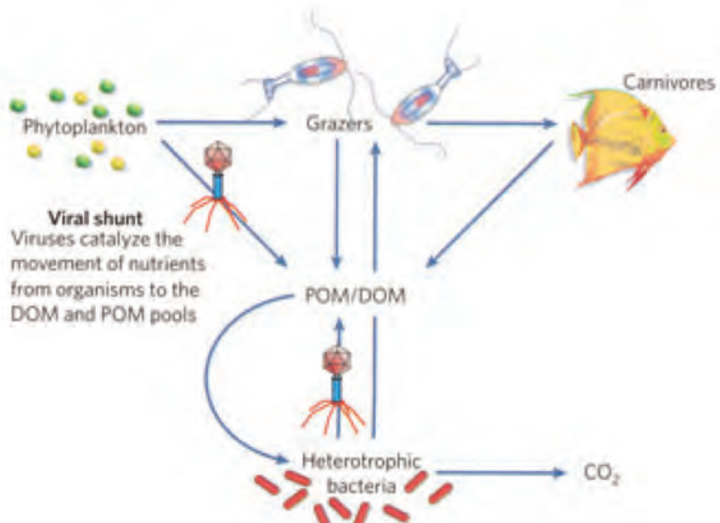


Figure 5.23 Viral influences on biogeochemistry. Viruses cause the lysis of cells, converting them into particulate organic matter (POM) and dissolved organic matter (DOM). This reduces the rate at which carbon sinks from the surface layer into the deep ocean where the carbon is trapped for millennia (biological carbon pump). Instead the carbon is retained in the surface waters where it is photooxidized and respired, in chemical equilibrium with the atmosphere. The net effect is a faster rate of CO_2 buildup in the atmosphere than would occur if the POM were "exported" to the deep ocean. (Reprinted by permission from Macmillan Publishers Ltd: *Nature*, from Suttle, C.A. 2005. Viruses in the sea. *Nature* **437**:356–361. Copyright 2005.)

evolution and biology is for specialized viral evolutionary lineages to infect autonomous self-replicating cells residing within each of the three domains of life: *Bacteria*, *Archaea*, and *Eukarya*. A shocking discovery in viral diversity has revealed that "virophages" exist: replicating virus particles can be parasitized! Sputnik is a novel virus (50 nm in size) whose host is the giant *Mimivirus* that infects the protozoan, *Acanthomoeba castellani* (La Scola et al., 2008). For additional information on viruses, see Granoff and Webster (2005), Cann (2012), Villarreal (2005), Knipe et al. (2001), Hurst (2000), Prangishvili (2013), and Norkin (2010).

5.9 MICROBIAL DIVERSITY ILLUSTRATED BY GENOMICS, HORIZONTAL GENE TRANSFER, AND CELL SIZE

As discussed in Chapter 3 (Section 3.2), the genetic blueprint of an organism, its genome sequence, is a comprehensive window into the evolutionary history and contemporary physiological ecology of the organism. We now reside in the genomic and postgenomic age. As of 2014, ~40,000 bacterial, ~1000 archaeal, ~10,000 eukaryal (fungi and protists), and ~4700 viral genome sequences had been completed, with many more in progress (US Department of Energy, Joint Genome Institute, http://img.jgi.doe.gov and http://www.ncbi.nih.gov/genomes/GenomesHome.cgi?taxid=10239&hopt=stat). Careful inspection of patterns within and between genomes (comparative genomics) is a new and novel tool for understanding life on Earth and the evolutionary processes that have operated. Comparative genomics, though still a rather young science, offers several key lessons about the diversity of microorganisms and traits shared between different taxonomic groups.

Genomic sizes of *Archaea*, *Bacteria*, *Eukarya*, and viruses do not sort into distinctive, orderly, predictable groups (Ward and Fraser, 2005; updates at http://www.ncbi.nih.gov/genomes/GenomesHome.cgi?taxid=10239&hopt=stat). Information portrayed in Figure 5.24 shows that some specialized members of the *Eukarya* (e.g., the protozoan parasite, *Cryptosporidium*, and the filamentous fungus, *Ashbya gossypii*) have streamlined genomes that roughly match the size of the larger bacterial genomes. Moreover, the photosynthetic protozoan ciliate, *Guillardia theta*, has a genome that is *smaller* than that of most bacteria. Perhaps, as expected, the sizes of bacterial and archaeal genomes have approximately the same range. However, some bacterial genomes (left-hand cluster in Figure 5.24) are very small (<1 Mb), reflecting highly specialized lifestyles. Other bacterial genome (right-hand cluster in Figure 5.24) are large, reflecting complex, diversified lifestyles. Remarkably, two viral genomes, those of the extremely large (400 nm) *Mimivirus* (that infects *Acanthamoeba polyphaga*; 1,181,549 bp) and *Cotesia congragata bracovirus* (CcBv; *Bracovirus* that infects the invertebrate *Cotesia congragata*; 567,670 bp), are as large or larger than several bacterial genomes (Ward and Fraser, 2005). Pandoraviruses, also hosted by acanthamoebae, have been discovered by Philippe et al. (2013) to have genomes as large as 2.5 Mb!

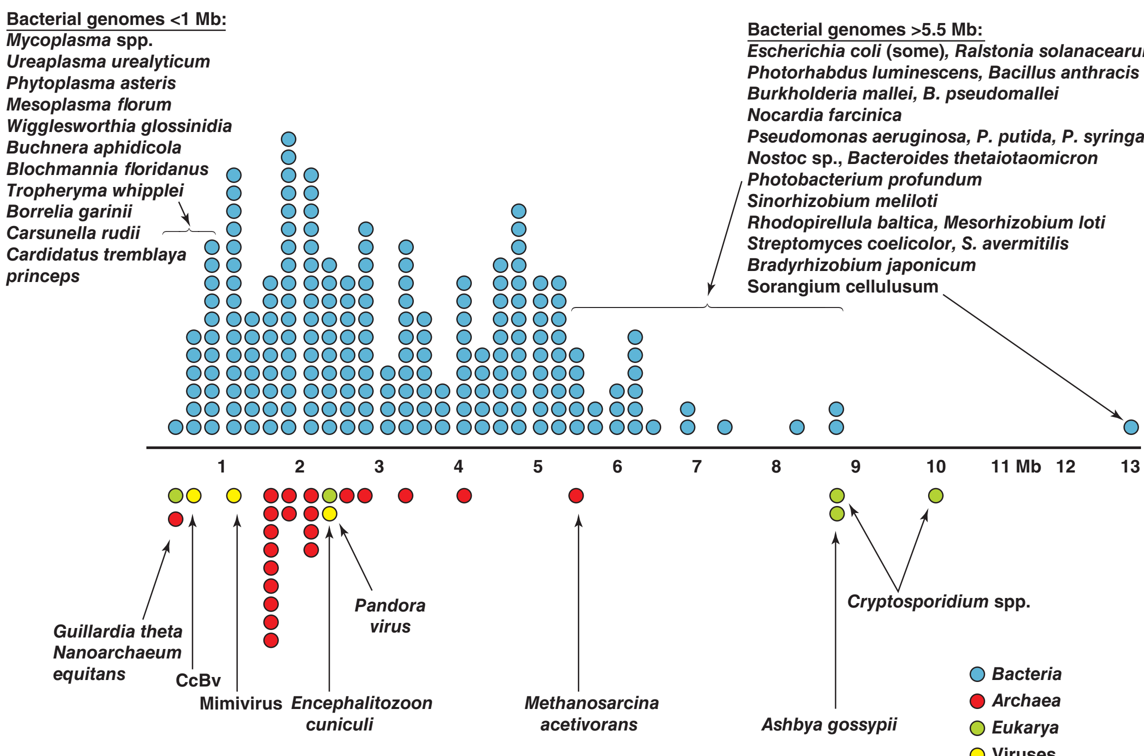


Figure 5.24 Depiction of overlapping genome size in members of the *Bacteria* (blue), *Archaea* (red), *Eukarya* (green), and viruses (yellow), in the size range (approximately 0.5–10.5 Mb) in which this overlap has been found to occur. The number of circles at a given point on the scale indicate the number of completed genomes that possess a specific size. Circles that represent unusually small (<1 Mb) or large (>5.5 Mb) bacterial genomes are labeled with the species name. (Reprinted and modified from Ward, N. and C.N. Fraser. 2005. How genomics has affected the concept of microbiology. *Curr. Opin. Microbiol.* **8**:564–571. Copyright 2005, with permission from Elsevier.)

Comparative genomics has also been a key contributor to the knowledge of horizontal gene transfer (HGT), the acquisition of heredity (especially DNA) from phylogenetically distant organismal lineages. In classic genetic theory, heredity elements (genes) are passed on "vertically" from generation to generation, from parental cell directly to progeny. HGT mechanisms (e.g., in prokaryotes the cellular uptake of DNA from the environment ("transformation"), intercellular plasmid transfer (conjugation), and virus-mediated genetic exchange (transformation)) are thought to contribute to genetic innovation in the organisms whose reproductive strategies lack eukaryotic sexual recombination. Through retrospective examination and comparison of genes among phylogenetically distant organisms, comparative genomics has made some startling discoveries. These include: (i) the presence of 81 archaeal-like genes clustered in 15 regions in the genome of

Thermotoga maritima (domain *Bacteria*; Nelson et al., 1999); (ii) the presence of a bacterial genome fragment from the insect endosymbiont, *Wolbachia*, in the X chromosome of the insect host (Kondo et al., 2002); (iii) the presence of bacteriophytochrome genes in the genome of the fungus, *Neurospora crassa* (Galagan et al., 2003); (iv) the human bacterial pathogen, *Legionella pneumophila*, genome contains 29 genes phylogenetically confirmed to be of eukaryotic ancestry (cited in Hottop, 2011); (v) expression in aphids of pigments encoded by carotenoid biosynthetic genes that are of fungal origin; and (vi) the presence of photosynthesis genes (photosystem I *psaA*) in viruses that infect cyanobacteria in marine ecosystems (cited in Hewson et al,. 2010). The means by which the above genes were exchanged remains largely unknown. Nonetheless, such HGT mechanisms are vital in contributing to biological diversity. The impact of HGT on both the form and validity of the tree of life has been hotly debated for many years (e.g., Doolittle, 1999; Gogarten et al., 2002; Lawrence and Hendrickson, 2005; Syvanen, 2012; Hottop, 2011). A version of the tree of life showing HGT is shown in Figure 5.25.

Another major trait for assessing biotic diversity is cell size (e.g., physical dimensions, shape, surface-to-volume ratio). This trait has major implications for the rate of material fluxes both within cells and between cells and their habitats. Ultimately cell size and shape reflect selective pressures over evolutionary history. Figure 5.26 presents a comparison of the size of known microorganisms (Ward and Fraser, 2005). Depicted on two different size scales are some of the smallest microorganisms (Figure 5.26, top) and some of the largest (Figure 5.26, bottom). The pattern in the range of physical sizes of organisms across domains is much like the pattern for genome sizes depicted in Figure 5.24. Representatives of *Eukarya*, prokaryotes, and viruses do not necessarily sort predictably by size: they have overlapping physical dimensions. The mimivirus particle is larger than the smallest *Mycoplasma* (a bacterial parasite that lacks a cell wall), while the Pandoravirus (Philippe et al., 2013) has dimensions that match those of the bacterium, *E. coli* (with a length of 1 μm; Figure 5.26). Continuing this counterintuitive trend in the dimensions of viruses, Legendre et al. (2014) recently isolated a novel, amoeba-infecting virus (*Pithovirus sibericum*) from Siberian permafrost measuring 1.5 μm in length! Moreover, at the other end of the size spectrum, the oxygenic phototroph, *Prochloron* (a symbiont of marine invertebrates), and the sulfur-oxidizing chemolithotroph, *Achromatium* (from freshwater sediments), are

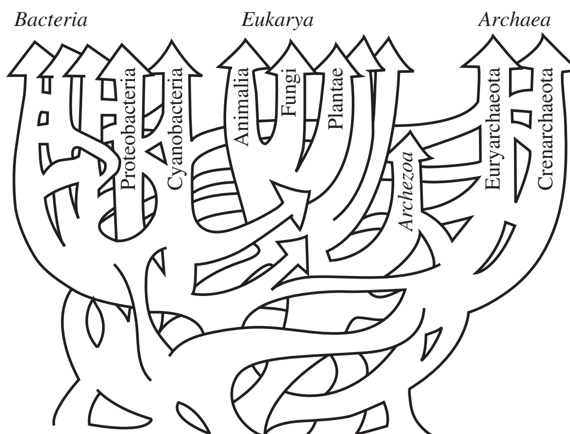


Figure 5.25 A version of the tree of life (based on small subunit rRNA sequences) that incorporates horizontal gene transfer processes in shaping the genetic composition of the three domains, *Eukarya*, *Archaea*, and *Bacteria*. (From Doolittle, R.F. 1999. Phylogenetic classification and the universal tree. *Science* **284**:2124–2129. Reprinted with permission from AAAS.)

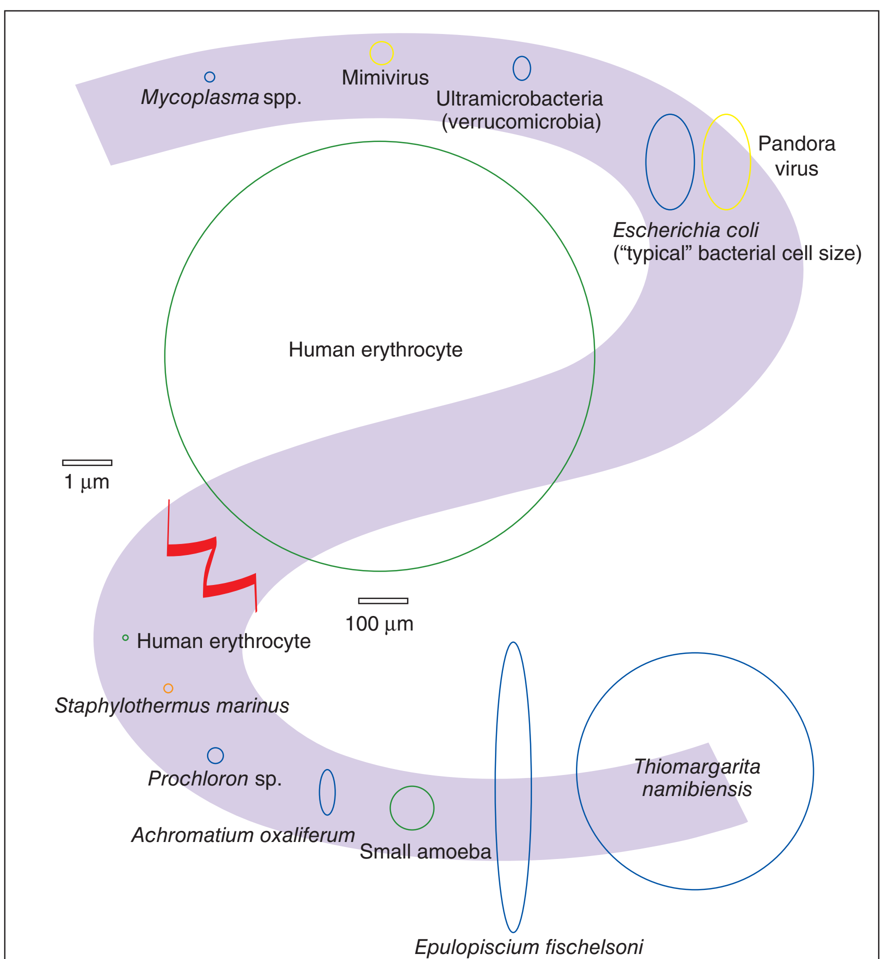


Figure 5.26 Depiction of overlapping cell size in members of the *Bacteria* (blue), *Archaea* (orange), *Eukarya* (green), and viruses (yellow). The diagram is divided into two different scales, with the upper portion showing the relative cell sizes of small and "normal" bacteria, as well as a mimivirus, in relation to a human erythrocyte (~9 µm in diameter). The same erythrocyte is used in the magnified lower portion of the diagram to demonstrate the large cell size present in certain bacteria, culminating in the extremely large cells (average 500 µm diameter) of the giant sulfur bacterium *Thiomargarita namibiensis*. (Reprinted and modified from Ward, N. and C.N. Fraser. 2005. How genomics has affected the concept of microbiology. *Curr. Opin. Microbiol.* **8**:564–571. Copyright 2005, with permission from Elsevier.)

much larger than a human blood cell (erythrocyte). Two prokaryotes are nearly unbelievably large: *Epulopiscium* (a chemosynthetic heterotroph from fish intestines) and *Thiomargarita* (a sulfur-oxidizing chemolithotroph) dwarf the two eukaryotic cells shown in Figure 5.26, the human blood cell (erythrocyte) and a small free-living amoeba.

5.10 BIOGEOGRAPHY OF MICROORGANISMS

Now that we have a grasp of the diversity of Earth's microbial inhabitants (*Bacteria*, *Archaea*, fungi, protists, and viruses; Sections 5.1 to 5.9), we are poised to address a critical new set of questions about environmental microbiology:

- Are there defined patterns of occurrence of microorganisms around the globe?
- "Who" do we expect to occur "where"?
- Are there analogies in the microbial world to polar bears in the Arctic, kangaroos in Australia, and giraffes in East and Southern Africa?
- What forces shape such distributions of microbial life? Shape their biogeography?
- What is known?

For nearly a century, ideas responding to the above set of questions have revolved around dogma attributed to two key scholars in environmental microbiology: M. Beijerinck and L.G.M. Baas Becking (see Section 1.3). The dogma has been "Everything is everywhere – the environment selects". Implicit in this statement are two premises: (i) the formidable dispersal capabilities of microorganisms (attributable to small size coupled to wind, water, and other transport vectors) has, over millions of years, resulted in a uniform "rain" of microbes across the globe and (ii) once alighting in aquatic, soil, or sediment habitats, habitat-specific resources (ranging from carbon source, nitrogen source, and electron acceptors to salinity, pH, and temperature to competition with other microorganisms) will allow only a small subset of the dispersed microorganisms to flourish, to take up residency.

It is safe to say that hundreds of scholarly articles have been published on the topic of microbial biogeography – some of these have been driven by "pure science" concerns about biodiversity and evolution; others by pressing practical concerns about disease transmission and refuges for pathogenic microorganisms. According to a recent review by Hanson et al. (2012), the key forces driving the varying patterns of abundance for microorganisms across Earth's habitats are: dispersal, selection, mutation, genetic drift, and historical legacy. The first two of these factors are identical to the two noted by M. Beijerinck and LG.M. Baas Becking. The second two factors, mutation and genetic drift, are processes that lead to genetic diversification of microbial populations after they have been dispersed into (and successfully colonized) a new habitat. "Historical legacy" acknowledges that prior conditions and resources in a given habitat (not simply contemporary properties) have shaped community composition. Naturally occurring microbial communities are composed of thousands-to-millions of populations. At a given point in time and space on our globe (e.g., your skin, the sea shore, a leaf on a tree, a farmer's field, sediments at the bottom of a lake), the composition of native microbial communities is determined by a dynamic balance between the five above-mentioned forces.

- **So what is known about *actual* microbial biogeographic patterns?**
- **Can such patterns be measured?**

Clearly, an ability to take a census of microbial life is fundamental to establishing biogeographic patterns. Sections 5.2 to 5.4 have raised the issue of census taking and this is expanded upon in Sections 6.7, 6.9, and 6.10. Building on the theme from Section 5.2, to discern biogeographic patterns, we must be able to recognize individual taxa or assemblages of taxa of interest, and then we must be able to compare the occurrences of such taxa (quantitatively or qualitatively) between different geographic sites. At many taxonomic levels (domain, phylum, order, family, genus, even species), microorganisms have been found to exhibit *cosmopolitan* distributions. For example, Ramette and Tiedje (2007) have cogently argued that the domains *Bacteria* and *Archaea* are globally distributed. Similarly, at the class level, *Betaproteobactera*, *Cyanobacteria*, *Flavobacteria*, and *Actinobacteria* routinely occur in aquatic and terrestrial sites worldwide. At the species level, many types of plant-pathogenic microorganisms threaten agricultural crops in global agriculture and *Pelagibacter ubique* is among many marine prokaryotes occurring across global seascapes (Ramette and Tiedje, 2007).

At the other end of the spectrum, highly restricted distributions of microorganisms (typically referred to as “endemic”) have been documented using fine levels of genetic resolution (such as species and ecotype). Endemism has been documented, particularly for microorganisms occupying relatively specialized ecological niches, such as *Rhizobium* (dwelling in plants and nodules) and *Sulfolobus* (dwelling in geothermal sites that are geographically isolated) (Ramette and Tiedje, 2007). Two relatively recent studies, below, provide a glimpse of current knowledge of microbial biogeography and of what is likely to appear in the future.

Nemergut et al. (2011) examined patterns within 28,115 sequences of 16S rRNA genes obtained from 14 different habitat types (ranging from estuarine, to sediments, to ice, to insect tissue, to lake water, to seawater, to soil) spanning 238 environmental samples. Each of the 28,115 sequences was classified taxonomically at four levels of sequence identity (98%, 95%, 92%, and 89%), considered by these authors to correspond roughly to “species”, “genus”, “family”, and “order”, respectively. Figure 5.27 provides a phylum-level overview of the identities of *Bacteria* native to this broad survey of natural habitats. In this pool of sequences, *Proteobacteria* were the most highly represented group (40%), followed by *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Fermicutes*, *Verromicrobia*, *Planctomycetes*, *Chloroflexi*, and *Cyanobacteria* (see Section 5.6 for descriptions of cultivated members of many of these phyla). For each of the 238 environmental samples, the distribution of sequences (taxa, identified at the four levels of identity, from “species” to “order”) was compiled into sample-specific assemblages representing community composition. A remarkable observation by Nemergut et al. (2011) was that, at the species

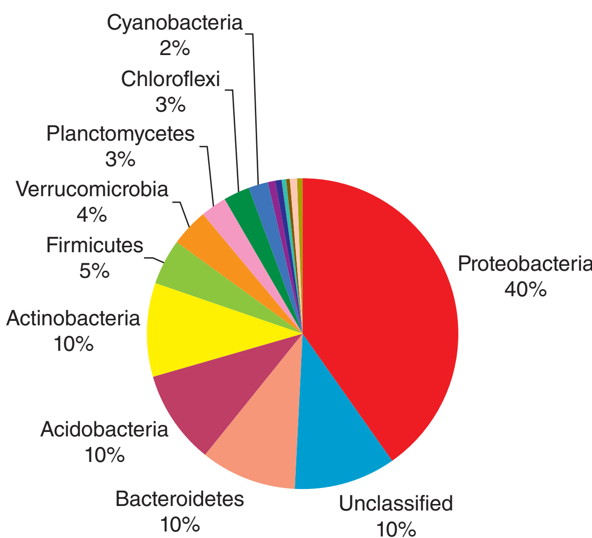


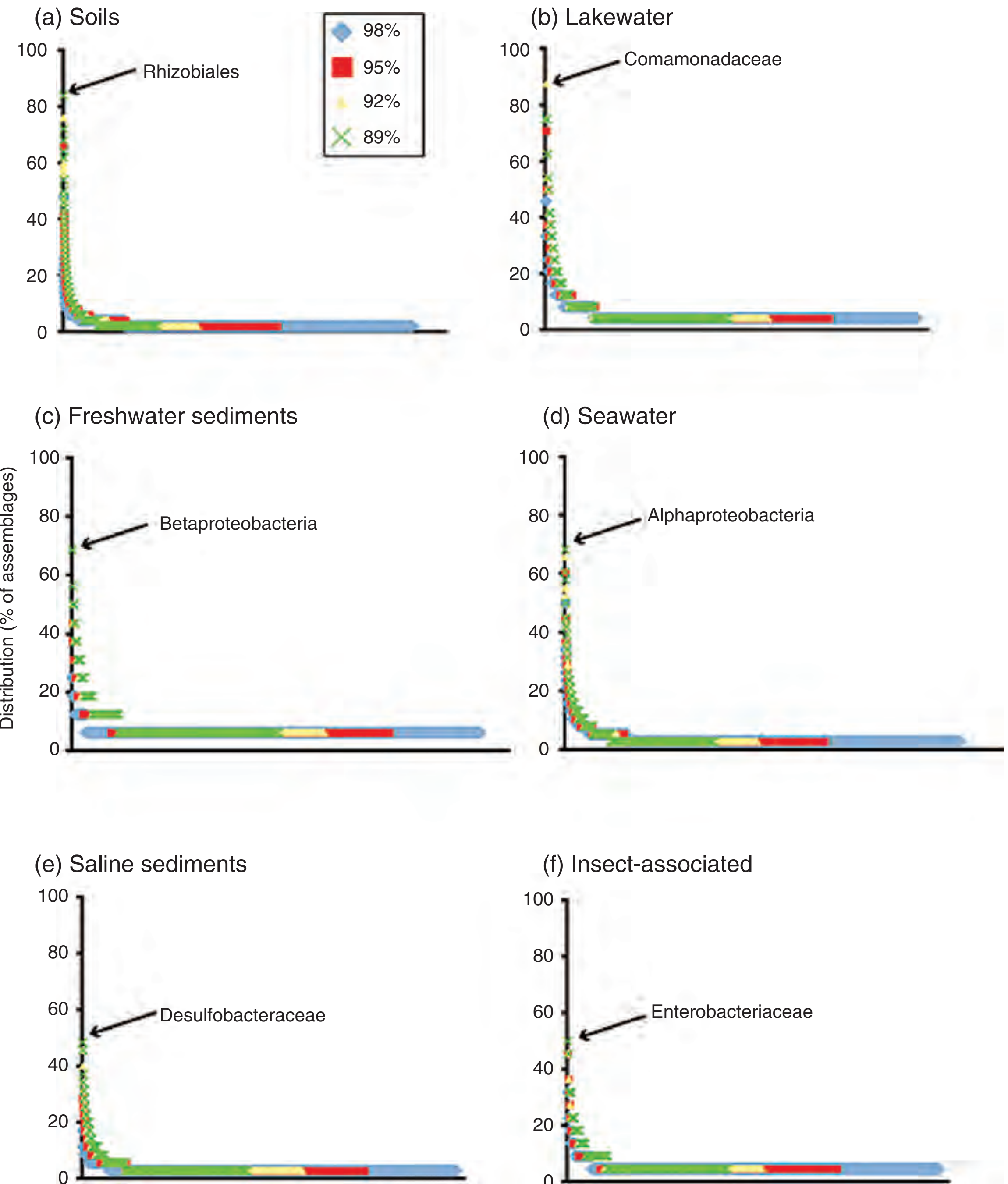
Figure 5.27 Survey of bacterial phyla found across many aquatic and terrestrial habitats. Shown is the relative abundance of different phyla within a clone library data set of 28,115 16S rRNA sequences obtained from 14 different habitat types (ranging from estuarine, to sediments, to ice, to insect tissue, to lake water, to seawater, to soil) spanning 238 environmental samples. Phyla that represent at least 2% of all sequences are labeled. (From Nemergut, D.R., E.K. Costello, M. Hamady, C. Lozupone, L. Jiang, S.K. Schmidt, N. Fierer, A.R. Townsend, C.C. Cleveland, L. Stanish, and R. Knight. 2011. Global patterns in the biogeography of bacterial taxa. *Environ. Microbiol.* **13**:135–144. Reprinted with permission from Wiley-Blackwell.)

level (the 98% level of discrimination between sequences), more than 85% of the sequences were found in only one community. Furthermore, no single sequence was found in more than 12% of the communities. When discrimination between sequences was relaxed to 89% ("order" level taxonomy), 35% of the classified sequences were found in two or more of the sampled microbial communities. However, at all levels of phylogenetic resolution, the majority of microbial taxa were found in only a single community. Thus, the data from Nemergut et al. (2011) support the concept that distinctive habitats support distinctive bacterial communities, whose members have limited distribution between habitats.

Another set of observations by Nemergut et al. (2011) supporting the above notion of "biogeographically distinctive community compositions" is presented in Figure 5.28. The prevalence of the most abundant taxa from six habitat types surveyed (soils, lake water, freshwater sediments, seawater, saline sediments, insect tissue) by Nemergut et al. (2011) are presented in Figure 5.28. The sequences were categorized at the same four levels of taxonomic discrimination (98%, 95%, 92%, and 89%) and then tallies for each of the categories were prepared and arranged from highest (left-hand side of each plot in Figure 5.28) to lowest (right-hand side of each plot). The main message from Figure 5.28 is that each habitat type was found to be dominated by distinctive bacterial populations.

Efforts to complete a global survey of marine microorganisms have been carried out by a large consortium of international marine microbiologists

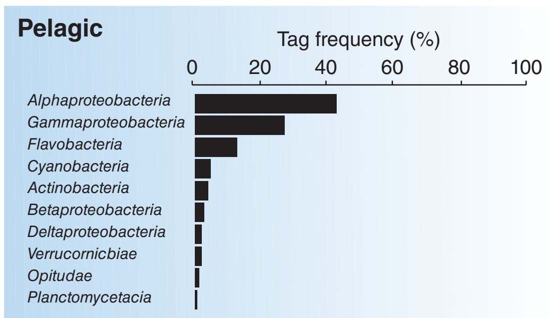
Figure 5.28 *(opposite)* Distinctive biogeograpic habitat types are dominated by distinctive microbial taxa (Operational Taxonomic Units, OTUs). The rank in distribution plotted against the percent of assemblages each OTU was found in for (A) soils (n = 49), (B) lakewater (n = 21), (C) freshwater sediments (n = 15), (D) seawater (n = 40), (E) saline sediments (n = 36), and (F) insect-associated samples (n = 15) for the clone library data. Those OTUs that were most widely dispersed within habitat types are indicated. Within habitat types, some OTUs were widely distributed among assemblages while the majority were limited to only a few assemblages. (From Nemergut, D.R., E.K. Costello, M. Hamady, C. Lozupone, L. Jiang, S.K. Schmidt, N. Fierer, A.R. Townsend, C.C. Cleveland, L. Stanish, and R. Knight. 2011. Global patterns in the biogeography of bacterial taxa. *Environ. Microbiol.* **13**:135–144. Reprinted with permission from Wiley-Blackwell.)

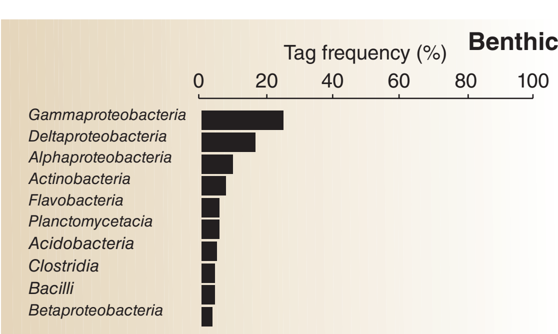

(a) Soils
Rhizobiales
98%
95%
92%
89%
(b) Lakewater
Comamonadaceae
(c) Freshwater sediments
Betaproteobacteria
(d) Seawater
Alphaproteobacteria
(e) Saline sediments
Desulfobacteraceae
(f) Insect-associated
Enterobacteriaceae
Distribution (% of assemblages)
Rank in distribution
100
80
60
40
20
0

(Zinger et al., 2011). The accruing data set, termed the International Census of Marine Microbes (ICoMM), has compiled measurements of naturally occurring microorganisms across global marine sites, with special emphasis on comparing water-column (pelagic) versus sediment (benthic) samples. Procedures used by Zinger et al. (2011) were similar to those used by Nemergut et al. (2011; see above), except that the 16S rRNA sequences were obtained, not from clone libraries but instead from next-generation 454 "pyrotag" sequencing (for details of this methodology, see Section 6.10). A total of 509 samples were sources of extracted DNA and 9.6 million sequences were analyzed. Results are portrayed in Figure 5.29. Despite the fact that pelagic (water column) and benthic (sediment) habitats are adjacent to one another and that the sediments accumulate materials settling from above, there are striking contrasts between the compositions of pelagic and benthic communities (Figure 5.29a): qualitatively there is no overlap among 40% (4 of the 10) of the most abundant bacterial classes. Moreover, for the six shared bacterial classes, there are major quantitative shifts. Thus, the sediment habitat clearly offers selective features (e.g., low oxygen availability, high organic carbon, microhabitat variations with a matrix of solid particles, etc.) that are distinctive from conditions in the water column.

To assess compositional contrasts between microbial communities across eight geographically distinctive pelagic and sedimentary marine sites, Zinger al. (2011) compiled 16S rRNA sequence abundances for 23 widespread marine taxa (Figure 5.29b). As shown along the bottom of Figure 5.29b, 5 pelagic ("P") realms and 3 benthic ("B") realms were sampled. Patterns in the quantitative prevalence in the 16S rRNA gene sequences of the 23 taxa are noted using a vertical arrangement of dots (large dots = high abundance; small dots = low abundance) for each of the 8 sampled habitats. Patterns in the dots describe microbial biogeography – they reveal commonalities and distinctions in microbial community composition between sites. Among the trends: *Alphaproteobacteria* are consistently abundant in marine open waters (not elsewhere); *Gammaproteobacteria*, though ubiquitous, are more prevalent in anoxic and vent-associated locales; also, the *Epsilonproteobacteria* appear to be uniquely suited to life in sediments adjacent to hydrothermal vents.

Figure 5.29 *(opposite)* A broad biogeographic survey of bacterial community compositions across marine habitats according to realms (water column versus sediment) and ecosystem types. A total of 509 marine samplers were sources of extracted DNA, leading to 9.6 million 16S rRNA gene sequences. (a) Average sequence frequency for the ten most abundant bacterial classes in the pelagic (water column) and benthic (sediment) realms. (b) Average proportions of the main bacterial taxa per realm and ecosystem type. P = pelagic, B = benthic. Notice that 23 taxonomic levels displayed on the right range from class to order to family – chosen to reflect widespread taxa whose ecology and diversity are commonly investigated in marine microbiology. (From Zinger, L., L.A. Amaral-Zettler, J.A. Fuhrman, M.C. Horner-Devine, S.M. Huse, D.B.M. Welch, J.B.H. Martiny, M. Sogin, A. Boetius, and A. Ramette. 2011. Global patterns in bacterial beta-diversity in seafloor and seawater ecosystems. *PLoS One* **6**(9):e24570. doi: 10.1371/journal/pone.0024570.g002. Reprinted with permission from www.plosone.org. Copyright Zinger et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.)





(b)

Alphaproteobacteria
Gammaproteobacteria
Flavobacteria
Deltaproteobacteria
Actinobacteria
Cyanobacteria
Betaproteobacteria
Planctomycetacia
Acidobacteria
Bacilli
Clostridia
Epsilonproteobacteria
Verrucornicrobiae
Sphingobacteria
Opitudae
Dehabcoccoidetes
Bacteroidia
Anaerolineae
Deferribacteres
Nitrospira
Deinococci
Spartobacteria
Aquificae

P-Coasial
P-Surface
P-Deep
P-Anoxic
P-Vents
B-Vents
B-Deep
B-Coastal

10%
30%
50%

STUDY QUESTIONS

1. The illustration in Figure 5.1 shows one way (DNA staining) to microscopically distinguish microbial cells from other particulate materials in environmental samples. Use your knowledge of biology to suggest additional ways of microscopic detection. Please state the approach, generally (based on broad principles of biomarker occurrence), and provide three specific examples or strategies.
2. Regarding uncultured organisms (Figures 5.2 and 5.3), the big challenge is trying to bridge the gap between "artificial laboratory media" and the metabolic resources that actually occur in nature. Can you suggest a way to create growth media that might successfully meet the challenge?
3. Envision this. You are the proprietor of a new bakery. The store offers many types of cookies. There are many variations on the themes of chocolate chip cookie recipes. Some have raisins, others oatmeal, others peanut butter, others are wheat-free, others are low calorie, others nondairy, while others offer mixed fruits, nuts, and butterscotch chips. You offer 250 permutations of the available combinations. In anticipation of the arriving crowds of customers, you begin to categorize the cookies so you can arrange and sell particular cookies from particular locations around the shop.
   (A) What signs would you make for displaying the cookies at each location? What criteria would you choose to distinguish one type of cookie from a close relative? Consider not only the ingredients in each recipe but the perceptions and expectations of the consuming public.
   (B) How different is this exercise from microbial taxonomy?
4. Section 5.4 and Box 5.3 describe six different approaches to assess microbial diversity. Devise a microbial diversity research question about a habitat that *you personally* are interested in. Then inspect the six diversity measurement approaches and choose one that best meets your objectives. Explain why you made your choice – justifying it on the basis of your goals, the assumptions behind the procedure, and the effort. Then state the limitations that you would need to understand when interpreting diversity data processed with the chosen approach.
5. In Section 2.7 the idea of a last universal common ancestor (LUCA) was introduced. Where is LUCA represented in Figure 5.7? What phylum shown on that figure is the closest relative to LUCA?
6. If, during evolution, humans had acquired chloroplasts and photosynthetic capabilities, where would we now fall on the tree of life (Figure 5.7)?
7. Information in Section 5.7 and Figures 5.15, 5.16, and 5.17 suggest that the rate of nonculture-based discovery of new organisms greatly exceeds the pace of culture-based discovery. What factors contribute to this situation? What developments may reverse it?
8. In Section 5.8, a "kill the winner" rule was mentioned as one of the ecological roles of viruses in natural habitats. Does this make sense to you? If so, why? If not, why not?
9. Write a paragraph on the potential impact of lytic bacterial viruses on biogeochemical processes. Include in your discussion the evolutionary logic of parasites killing their hosts.
10. Figure 5.26 shows bacteria that are thousands of times larger than a common *E. coli* cell. Given the discussion of cell size in Section 3.5, can you make any inferences or develop testable hypotheses about the selective pressures, habitat, and bacterial physiologies that lead to large bacterial cells?
11. One of the blatant trends in the biogeography patterns shown in Figure 5.29 is enrichment of *Epsilonproteobacteria* in sediments adjacent to hydrothermal vents. Based on this survey of taxonomic (16S rRNA) genes, carry out 4 tasks: (i) formulate a hypothesis about why *Epsilonproteobacteria* may flourish at vent sites; (ii) state the physical conditions, key environmental parameters, and physiological resources for vent sites; (iii) state several key physiological and metabolic traits that microorganisms dwelling in such sites would likely need to make a living there; and (iv) conduct a search in the current microbiology literature (noting sources) that empirically verify that the adaptations you hypothesized *might* be useful are *truly used* by *Epsilonproteobacteria*.

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6

Generating and Interpreting Information in Environmental Microbiology: Methods and Their Limitations*

Using routine human curiosity as a guide, the standard set of questions to pose about naturally occurring microorganisms that dwell in biosphere habitats (e.g., water, sediments, soils, gastrointestinal tracts of animals, and all of the remainder) are: "Who? What? When? Where? How? Why?" Information in Chapter 5 (see Section 5.2) introduced the reader to the broad essentials of the methodological approaches in environmental microbiology (see Box 5.1) and to census taking – especially as it applies to microbial biogeography (see Section 5.10). This chapter expands and elaborates upon the details of measurement procedures, experimental designs, data generation, and data interpretation. These details are essential for ongoing and future efforts aimed at advancing understanding of environmental microbiology, itself, and of its related disciplines (e.g., environmental engineering, biogeochemistry, ecology, medical microbiology, biotechnology). There are a variety of useful methods-related references that are available (e.g., De Bruijn, 2011a, 2011b; De Bruijn, 2013; Hurst et al., 2007; Kowalchuk et al., 2004; Osborne and Smith, 2005).

Environmental microbiology is a methods-limited discipline. In this book, we have already seen at least two concrete examples of methodological innovations that drastically changed the intellectual landscape of environmental microbiology: (i) the development of nonculture-based procedures (see Sections 5.1, 5.2, 5.4, and 5.7) and (ii) Woesean molecular phylogeny, which redefined evolutionary and taxonomic relationships among microbes (see Section 5.5). Many investigators would argue that metagenomics (Section 6.9) represents another major methodological innovation. Furthermore, there is growing sentiment that the most recent (twenty-first[t] century) innovation in Environmental Microbiology is the application of "Omics" technologies (e.g., genomics, transcriptomics, proteomics, metabolomics) to the analysis of naturally occurring microbial communities in real-world habitats (e.g., seawater, soil, the human gut; see Section 6.10). There is every reason to anticipate additional methods-related paradigm shifts in environmental microbiology's future. Both Konopka (2006) and Prosser et al. (2007) have argued that a unifying set of theoretical ecological principles would be immensely beneficial in guiding future advancements in microbial ecology and environmental microbiology – such theories have the potential to match or exceed the influence of methodological innovation.

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Chapter 6 Outline

6.1 How do we know?
6.2 Perspectives from a century of scholars and enrichment-cultivation procedures
6.3 Constraints on knowledge imposed by ecosystem complexity
6.4 Environmental microbiology's "Heisenberg uncertainty principle": model systems and their risks
6.5 Fieldwork: being sure sampling procedures are compatible with analyses and goals
6.6 Blending and balancing disciplines from field geochemistry to pure cultures
6.7 Overview of methods for determining the position and composition of microbial communities
6.8 Methods for determining in situ biogeochemical activities and when they occur
6.9 Cloning-based metagenomics and related methods: procedures and insights
6.10 Cloning-free, next-generation sequencing and Omics methods: Procedures and insights
6.11 Discovering the organisms responsible for particular ecological processes: linking identity with activity

6.1 HOW DO WE KNOW?

Look out the window. Stroll through a woodland or a garden. Scrutinize photographs of the Earth's various biomes. What is happening in those habitats from a biogeochemical standpoint? How do we know what is happening? Box 6.1 illustrates what we can and cannot directly infer from snapshot images of landscapes. Environmental microbiology is a multifaceted discipline that relies upon natural history, inference, deduction, and experimentation to draw conclusions about causality in our world.

Epistemology is a way to approach information and knowledge. *The Oxford Companion to Philosophy* (Honderich, 1995) defines epistemology as "that branch of philosophy concerned with the nature of knowledge, its possibility, scope, and general basis". However, epistemology does not exist only in philosophical realms. Six decades earlier, Cunningham (1930) defined epistemology as "the science which sets forth and establishes the existence of true and certain human knowledge, the means of acquiring such knowledge, and the norm by which we can distinguish such knowledge from falsity". In forging even stronger bonds between scientific inquiry and epistemology, Bateson stated that epistemology is "a branch of biology… [it is] the process of the acquisition of information and its storage" (Donaldson, 1991). It is "the science of how we can know anything". Despite the clear impetus for pursuing knowledge of environmental microbiology, throughout its history, methodological limitations have impeded obtaining answers to fundamental questions about microorganisms in the biosphere. The objectives of this chapter are to examine constraints on knowledge of environmental microbiology and to describe how an integration and accrual of new methodologies into a continuum of field and molecular observations progressively advances the epistemological basis of environmental microbiology.

6.2 PERSPECTIVES FROM A CENTURY OF SCHOLARS AND ENRICHMENT-CULTIVATION PROCEDURES

Environmental microbiology may be described as a science for optimists. Driven by a sense of significance and discovery, investigators forge ahead despite adversity. One way to appreciate the role of optimism in environmental microbiology relies upon the metaphor of half a cup of green tea.

Box 6.1

A typical ecosystem: how do we know what biogeochemical processes are occurring?

(Courtesy of E.L. Madsen, Cornell University, with permission.)

Left is a photograph of a temperate forest and freshwater stream. What organisms are there? What is happening biogeochemically? Is carbon being fixed? Is carbon being respired? Is nitrogen being fixed? Is the nitrogen in biomass proteins being converted to amino acids, then ammonia, then to nitrate? Is sulfur being cycled? What organisms are there? Plants? Protozoa? *Archaea? Bacteria*? What are they doing? The only definite biogeochemical process evident in the photograph is CO_2 fixation by plants. Without photosynthesis, the plants would be absent.

Illustrating the methodological challenges: higher plants and their biogeochemistry versus microorganisms and theirs

The methodological challenges of discerning microbial activities in soil, sediment, or water may perhaps best be appreciated by considering how we know that higher plants carry out photosynthesis. In surveying a given landscape, humans can gather evidence for photosynthesis simply by noting the location of vegetation. Humans and the vegetation are roughly the same scale (approximately meters); therefore, detecting plants and their spatial relationships to one another and their habitats is facile. Photosynthesis is the major biogeochemical function of higher plants; without it there would be no plants, or food chains based thereupon. Thus, the presence of higher plants provides evidence for conversion of atmospheric CO_2 to biomass and (because rooted plants are immobile) we simultaneously discern where the photosynthesis has occurred. At a mere glace, then, humans gain plant-related biogeochemical knowledge addressing four key questions: "who?" (the plant), "what?" (photosynthesis), "when?" (recent history), and "where?" (the plant's location). To gain knowledge of the remaining two commonly asked key questions, "how?" and "why?", we rely on reductionistic biological disciplines that include physiology, biochemistry, genetics, and molecular biology – some of which can be applied to field-gathered plant samples or be manifest as chambers deployed to field sites. There are many striking contrasts between how the six key questions pertinent to plant photosynthesis are answered and how the same questions are answered for metabolic activities of microorganisms in field habitats (see main text).

Pessimists are often defined as the type of people who complain about the cup being half empty. Optimists, on the other hand, emphasize that the cup is half full. In environmental microbiology, research efforts seldom lead to a *complete* data set. There is always some missing information and the missing portion may be immense. The quotes in Table 6.1 provide a historical perspective on environmental microbiology's long-standing methodological limitations. The first entry in Table 6.1 is from S. Winogradsky, who worked extensively from the late 1800s into the 1900s (see Section 1.3). The views shown in Table 6.1 span a century and show at least two major themes: (i) traits of microorganisms expressed in the laboratory are not likely to be those found in nature and (ii) methodological obstacles need to be overcome so that long-standing answers to ecological questions can be obtained.

Enrichment culturing from nature tells us what might happen. Some of the earliest and most influential investigations in the history of environmental microbiology relied on enrichment culturing strategies (Beijerinck, 1888; Winogradksy, 1949; Overmann, 2006) to identify and isolate individual microbial cultures capable of carrying out novel metabolic processes, such as growth on ammonia as an energy source, fixation of atmospheric nitrogen into cell protein, and the use of unusual (perhaps pollutant) organic compounds as carbon and energy sources or as final electron acceptors.

Enrichment culturing uses a sample of a naturally occurring microbial community as an inoculum for laboratory-prepared growth medium that is designed to select a small subset of the initial community (Figure 6.1). The logic behind enrichment culturing involves devising growth conditions that allow particular members of the community to multiply and eventually dominate within the mixed populations that were initially present. For

Table 6.1

Historical excerpts documenting methodological limitations in environmental microbiology (copyright 1996, from Madsen, E.L. 1996. A critical analysis of methods for determining the composition and biogeochemical activities of soil microbial communities *in situ*. In: G. Stotzky and J.-M. Bollag (eds), *Soil Biochemistry*, Vol. 9, pp. 287–370. Reproduced by permission of Taylor and Francis Group, a division of Informa plc.)

Excerpt	References
"We have as yet no science of soil microbiology proper, although we possess a great deal of information on various groups of soil microorganisms and what they can do when grown in artificial culture media", said S. Winogradsky, whose exact contributions to our understanding of certain soil microbiological processes can hardly be equaled.	Waksman, 1927
We possess, at the present time, considerable information on the organisms inhabiting the soil and on the chemical processes of many of these organisms, under controlled laboratory conditions; but little is known of the processes carried out in the soil itself, by the numberless representatives of the soil flora and fauna.	Waksman, 1927

Table 6.1 *Continued*

Excerpt	References
When I stand on the litter of a forest floor, or on a thatch of grassland, I like to look down, to ask myself just what do I really know about the microorganisms in that ecosystem. Immediately a whole host of categorical relationships, or definitions and generalizations from textbooks of ecology, come to mind. I feel a certain satisfaction in knowing that moisture and temperature influence microbial activity, but nevertheless, I still feel unable to comprehend the total functional ecology in that ecosystem. I know with reasonable assurance what measurements I can make or what microbes I can isolate or enumerate or identify within my own limited area of competence, and I realize what an exceedingly small area that competence embraces.	Clark, 1973
But, however the organism is obtained, whether from nature or from the laboratory, it is clear that it is not sufficient that the organism or consortium of organisms act . . . in the laboratory. Action must occur in the world at large. If we have learned nothing else from research in microbial ecology, we have learned that microbes do not do the same things in the laboratory that they do in nature.	Brock, 1987
The composition of the microbial community is still uncertain. The old explanations for the low recoveries of bacteria by plate counts and the platitudes concerning the role of particular morphological or physiological groups in soil processes remain.	Alexander, 1991
The stunning impact that new methods are having on aquatic microbial ecology points out that the field is still methods limited. Despite tremendous progress over the last 25 years, we lag well behind other ecological research areas. We have only a primitive ability to describe the organisms present in nature, what these organisms are actually doing, and what controls their activity and growth.	Hobbie, 1993
Both the activity and biomass measurements can be artifacts of the experimental methods employed. To some degree, the whole field is still methods limited. There are many successful methods but we still lack some crucial techniques that would allow a complete picture. Much of the effort in the field has been spent on investigations of the physiology and potentialities of many types of bacteria in the laboratory, yet in the field we do not even know the identity of the species carrying out many of the important processes, or the correct rate of growth of bacteria in water and sediments.	Hobbie and Ford, 1993
We need information on the species carrying out the microbial processes, the controls of these processes, the importance of grazing by protozoans or infection by viruses as controls of the microbial biomass, and microbial growth rates in nature. Although there has been tremendous progress, key techniques for measurement are still needed before we can have faith in the results and begin to apply the techniques over a range of habitats. These techniques will allow a quantitative view of the ecology of aquatic bacteria rather than the largely qualitative and descriptive view we have today.	Hobbie and Ford, 1993
The development of new methodologies to understand microorganisms in their environments has become a science unto itself. The need for these methods comes from the fact that classic methodologies of medical and industrial microbiology developed over the last hundred years produce artifacts when applied to natural populations in the environment.	Paul, 1993

instance, if one is interested in finding aerobic microorganisms that can grow on benzene (oxidizing it to CO_2 and incorporating the substrate carbon into new cells), then the enrichment medium would contain benzene as the sole carbon and energy source, and oxygen as the electron acceptor. A 1 g soil inoculum can contain thousands of species (see Section 5.4), although only a small percentage of these would be expected to grow on benzene. After a 1–2-week incubation, benzene degraders would become dominant. Then, by plating small volumes of the enriched populations on to benzene growth medium solidified with agar (Figure 6.1), individual colonies of benzene degraders can be picked, further purified, isolated, and characterized using appropriate physiological, biochemical, and/or genetic procedures. *It is important to note that the microorganisms found from enrichment culture procedures and the metabolic information they generate may be ecologically irrelevant.* Each microorganism (in the laboratory or in nature) has the genetic potential to carry out a multitude of metabolic processes – each of which is conditionally regulated by their environment. Therefore, the presence in an environmental sample of a particular organism or gene that is capable of catalyzing a particular process cannot be taken as evidence that the process is occurring in situ (Brock, 1987).

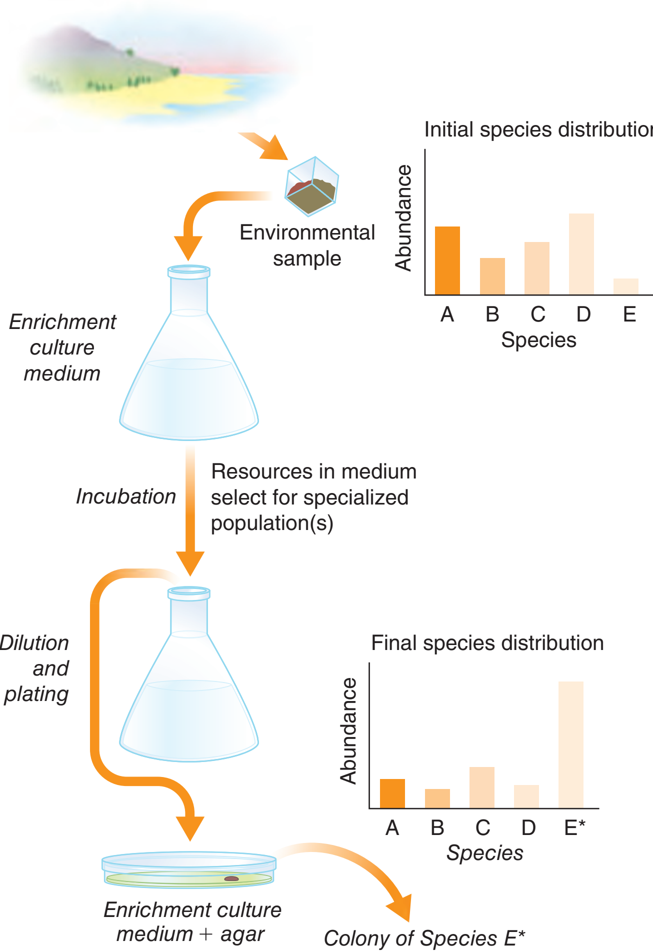


Figure 6.1 Enrichment culturing procedures. Growth conditions in the enrichment-culture medium provides electron donor, electron acceptor, and nutrients that allow a subset of the initial microbial community to flourish. To illustrate population changes during enrichment, five hypothetical species (A through E) are shown before and after enrichment.

6.3 CONSTRAINTS ON KNOWLEDGE IMPOSED BY ECOSYSTEM COMPLEXITY

To know that microorganisms are the agents of geochemical change in soil, sediments, and waters, environmental microbiologists face the challenge of documenting both the change (e.g., conversion of plant biomass to CO_2 in sediments, nitrogen fixation in soil, or methane production in wetlands)

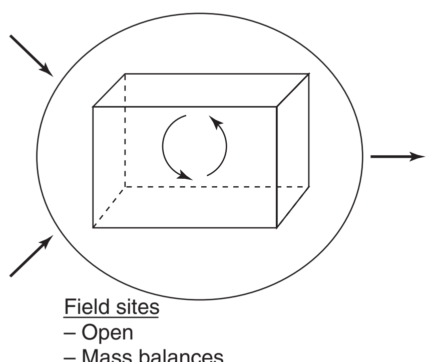


Figure 6.2 Diagram illustrating the open, dynamic nature of real-world microbial habitats such as soils, sediments, and waters. Not only do materials flow through the systems (making mass balances difficult to assemble), but the materials can be consumed, or generated, by biological or chemical processes with this system.

and the role of microorganisms as causative agents. *Microorganisms live and act in aquatic and terrestrial habitats whose complexities pose obstacles that impede directly measuring in situ activities of residence microorganisms*. As described in Chapters 1 and 4, these are continuous, open systems subject to fluxes of energy (e.g., sunlight, wind, tides) and materials (e.g., aqueous precipitation, erosion, deposition, infiltration, runoff) (Figure 6.2; see also Figures 1.3 and 1.4). Thus, accurate accounting of the masses of materials is difficult, if not impossible, in most habitats. Even if accounts of material fluxes through open systems were accurate, another task remains – distinguishing microbial activities from the many other processes (chemical, physical, and physiological or other transformations carried out by higher organisms) that also influence field geochemical parameters. Except for photosynthesis by higher plants, most physiological reactions carried out by higher organisms are of little global significance. Yet in many localized habitats the contribution by plants and animals to the production, consumption, and transformation of geochemical materials cannot be ignored.

Although interactions within food webs can be modeled (see Figure 1.4), comprehensive documentation of the many simultaneous nutrient cycling, trophic, and biochemical interactions in field sites has yet to be achieved (Parton et al., 1988; Kroeze et al., 2003; Manzoni and Porporato, 2009; McGuire and Tresender, 2010). Physical and chemical (abiotic) processes that must also be considered when measuring geochemical change in field settings include dilution, advection, dispersion, volatilization, sorption, photolysis, alteration by clay surfaces or other inorganic materials, and inorganic and organic equilibria (Voudrais and Reinhard, 1986; Wolfe, 1992; Thibodeaux, 1995; Stumm and Morgan, 1996; Schwarzenbach et al., 2002). Furthermore, within each microbial habitat, complex synergistic geochemical changes are effected by consortia of microbial species (Schmitz et al., 2006; Sieber et al., 2012; Zinder, 1993; Schink and Stams, 2006; see Section 3.10). Additional complexity of microbial activities in field sites stems from their dynamic changes in time and space (see Section 1.4). The physical, chemical, nutritional, and ecological conditions of microorganisms in field settings are heterogeneous and vary from the micrometer to beyond the kilometer scales (Groffman, 1993; Hobbie, 1993; Hobbie and Ford, 1993; Parkin, 1993; Manzoni and Porporato, 2009; Hartman and Richardson, 2013; Yates and Warrick, 2012). Moreover, the biota and their respective physiological (e.g., growth rate, excretion, differentiation, death) and behavioral (e.g., migration, predation, competition, parasitism, symbiosis; see Sections 8.1 and 8.2) activities respond to climate-induced and/or other environmental changes. As mentioned in Chapter 1 (Section 1.4), if concentrations of any key biogeochemical parameters (e.g., ammonia,

nitrate, methane, dissolved organic carbon) are found to fluctuate in lake water or sediments, interpreting such field measurements is very difficult. The changes in nutrient pools at any given moment are controlled by processes of production, consumption, and transport. Clearly, the many compounded intricacies of field habitats and microorganisms make their geochemical activities difficult to decipher.

6.4 ENVIRONMENTAL MICROBIOLOGY'S "HEISENBERG UNCERTAINTY PRINCIPLE": MODEL SYSTEMS AND THEIR RISKS

A Heisenberg-type uncertainty principle is inescapable in environmental microbiology (Madsen, 1991, 1998a, 2005) and must be confronted both in the examination of field-site samples and in exploiting the spectrum of disciplines that contribute to our mechanistic understanding of microbiological processes. When one begins in a field site or with site-derived samples, *the closer microorganisms are examined, the more likely the resultant information is to suffer from artifacts imposed by the measurement procedures*. This dilemma, which acknowledges the linkage between performing measurements on microorganisms and imposing artifacts on resultant data, is analogous to the Heisenberg uncertainty principle in quantum chemistry (Castellan, 1983; Zumdahl, 1986; Madsen, 1991). According to quantum theory, accurate measurements of the position and momentum of an electron are mutually exclusive. In environmental microbiology, high precision in reductionistic procedures and the relevance of information derived therefrom to field biogeochemical processes are often mutually exclusive because artifacts may develop. The basis for such artifacts is habitat disturbance and the responsiveness of both individual microorganisms and entire microbial communities to environmental change implicit in habitat disturbance (Hobbie, 1993; Hobbie and Ford, 1993; Madsen, 1996). Microorganisms are small (on the order of micrometers). The million-fold discrepancy in size between humans and microorganisms ensures that gathering field samples for microscopic and other analyses will physically disturb both the microorganisms and their habitats. Environmental microbiologists generally agree that, given sufficient time, the microbial community present in every environmental sample will change according to selective pressures (resources and environmental conditions) imposed by the removal of samples from their original location in field sites and by all intentional and unintentional laboratory incubation conditions (temperature, oxygen tension, physical disturbance, addition of nutrients or growth substrates, etc.). Removal of samples from a study site is equivalent to embarking on enrichment culturing procedures.

As described in Section 6.2, implicit in enrichment culturing procedures is the ability of microorganisms to respond and change when subjected to environmental perturbations. The nature of the microbial responsiveness

during enrichment culturing is clear: resuscitation from dormancy and growth of (often) minor populations during laboratory incubation periods lasting days to years. However, even if relatively brief incubations preclude shifts in population dynamics owing to growth and death, microorganisms still respond to environmental change. For instance, intricate biochemical signaling pathways allow cells to sense and respond to key nutrients (e.g., light, O_2, other electron acceptors, carbon sources; Antelmann et al., 2000; see Section 3.5), stress (e.g., acid, oxidative damage, inhibitory substances; Imlay, 2003; Chiang and Schellhorn, 2012), and cell-to-cell signaling molecules (quorum sensing pheromones; Rutherford and Bassler, 2012; Ng and Bassler, 2009; Schuster et al., 2013; see also Section 8.4 and Box 8.9). Timeframes for these responses range from nanoseconds (light), to milliseconds (O_2, toxicity), to minutes (enzyme synthesis) or hours (sporulation) (see Section 3.5, Table 3.5, and Figure 3.7).

This remarkable propensity of populations within naturally occurring microbial communities to change is a blessing for microbiologists practicing enrichment culture. However, it is a major impediment for those seeking to interpret physiological and ecological measurements performed on laboratory-incubated environmental samples such as water, soil, or sediments. The validity of measurements conducted on microbial communities removed from their original field setting is uncertain, because we cannot be sure that conditions imposed upon the native microorganisms (postsampling and incubation) have not quantitatively or qualitatively altered their populations and physiological reactions. Potentially misleading "bottle effects" are implicit in all measurements performed on sampled microbial communities moved from their place of origin to a new, contrived place where measurements can be completed (e.g., Venrick et al., 1977; Vaulot et al., 1995).

Therefore, for the practicing environmental microbiologist, there is considerable controversy surrounding the amount of time required for microorganisms in environmental samples to respond to sampling and experimentally induced environmental changes. Implicit in many published investigations is the hypothesis that accurate qualitative and quantitative microbial activity determinations of in situ processes can be performed in the laboratory within a "safe period" before artifacts develop (Figure 6.3). This hypothesis has not been adequately tested, yet its validity is essential for the extrapolation of results from laboratory incubations to field sites (Staley and Konopka, 1985; Karl, 1986, 1995; Tiedje et al., 1989; Pinckney et al., 1995; Madsen, 1996, 1998a). The alternative (conservative) methodological approach views laboratory incubations of environmental samples, at best, as a means toward estimating field processes. From the conservative viewpoint, quantitative extrapolation from laboratory results to actual field processes is taboo (Karl, 1995; Madsen, 1998a) because the instant an environmental sample is removed from a field study site, intricate and tightly regulated genetic-, biochemical-, cellular-, and population-level changes may be triggered (see above). It is the investigator's

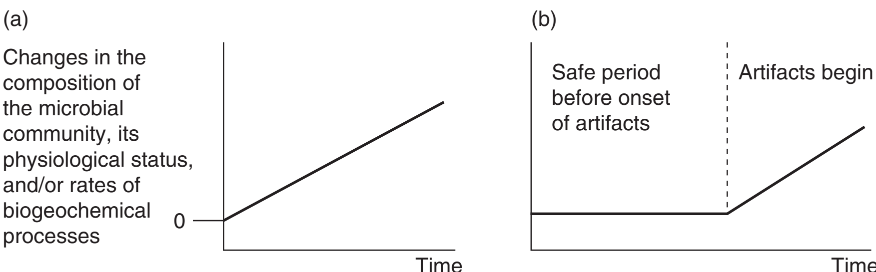


Figure 6.3 Uncertainties in seeking data on in situ biogeochemical processes from samples removed from the field and incubated in the laboratory. The two graphs describe the quantitative and/or qualitative influence of sampling and incubation on biogeochemical processes of interest. (a) Changes in environmental samples may begin the instant they are disturbed in a field site or (b) after some uncertain "safe period" during which valid measurements may theoretically be completed. (From Madsen, E.L. 1996. A critical analysis of methods for determining the composition and biogeochemical activities of soil microbial communities *in situ*. In: G. Stotzky and J.-M. Bollag (eds), *Soil Biochemistry*, Vol. 9, pp. 287–370. Copyright 1996. Reproduced by permission of Taylor and Francis Group, a division of Informa plc.)

inability to obtain disturbance-free samples and to fully characterize, understand, and duplicate field conditions in the laboratory that undermine the acceptance of laboratory measurements performed on field samples as valid surrogates for true in situ field processes. Figure 6.1 illustrates how the abundances of microbial populations (and hence, their physiological reactions) can shift during laboratory incubation of environmental samples, even in the absence of selective media.

6.5 FIELDWORK: BEING SURE SAMPLING PROCEDURES ARE COMPATIBLE WITH ANALYSES AND GOALS

Sampling is a scientific activity whose importance cannot be overestimated. The most sophisticated methodologies asking "Who? What? When? Where? How? Why?" deliver erroneous information if environmental samples containing natural microbial communities are gathered improperly or mishandled.

Proper sampling of the source of microorganisms of interest is critical for achieving valid data in environmental microbiology. Results from microscopic, biomarker, physiological, and cultivation protocols described in this chapter are only as sound as the investigators' hands in gathering microorganisms that truly represent the sampling site. Aseptic techniques (such as the use of flame-sterilized implements and the enclosure of samples within previously sterilized vessels) are often essential. Because microbiological

characteristics of environmental samples are prone to postsampling changes (see Section 6.4), fixation procedures should be carefully scrutinized.

The overarching theme in sampling microbial habitats and in subsequent handling of the samples is to be sure that postsampling procedures minimize changes in the microbial community while affording acquisition of the sought information. Figure 6.4 provides an overview of the four procedural steps that lead from a field site of interest to information about the native microorganisms. The capture–fix–store–analyze scheme in Figure 6.4 goes hand-in-hand with the paradigm shown in Figure 6.5, which integrates the questions posed with both the methods used and the corresponding sampling procedure.

Sampling for microbiological analyses

As shown in Figure 6.5, the environmental microbiological questions being posed, the methods being used, and the information being generated are

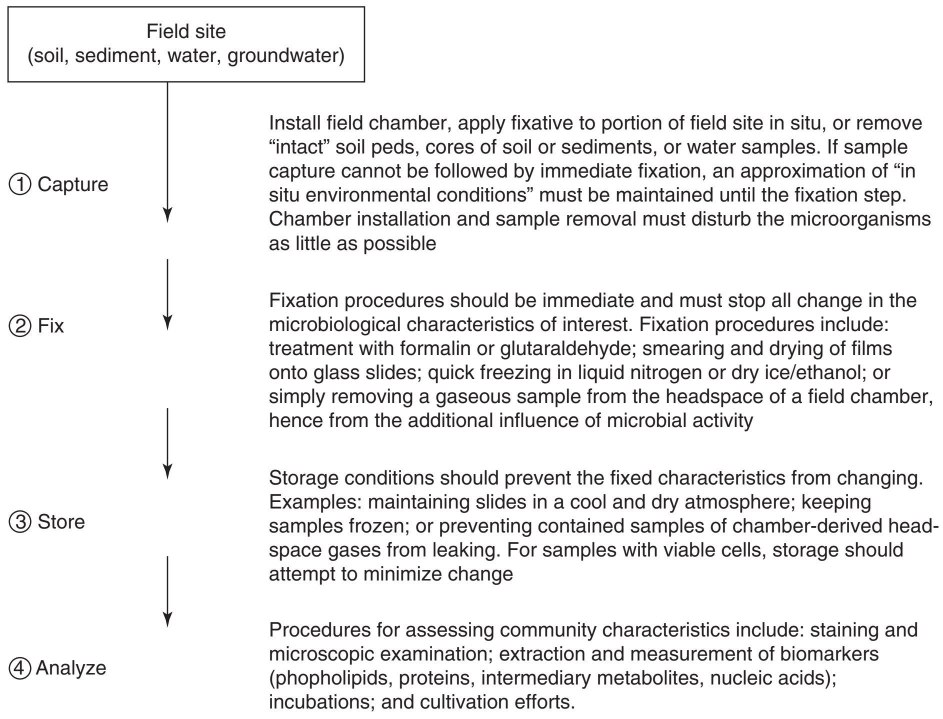


Figure 6.4 Four-step methodological scheme for sampling and processing and generating information from microbial communities in nature. (From Madsen, E.L. 1996. A critical analysis of methods for determining the composition and biogeochemical activities of soil microbial communities *in situ*. In: G. Stotzky and J.-M. Bollag (eds), *Soil Biochemistry*, Vol. 9, pp. 287–370. Copyright 1996. Reproduced by permission of Taylor and Francis Group, a division of Informa plc.)

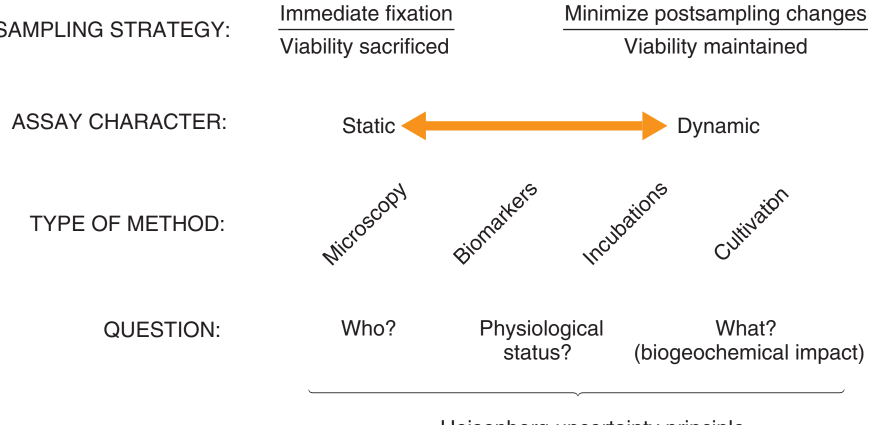


Figure 6.5 Integrated paradigm for methodological approaches in environmental microbiology. The four basic types of methods (microscopy, biomarkers, incubations, cultivation) are shown in relation to the questions being asked, to the character of the assays (static versus dynamic), and to the appropriate sampling strategies. Environmental microbiology's Heisenberg uncertainty principle (see Section 6.4) appears at the base of the diagram because avoidance of methodological artifacts is crucial in the quest to obtain valid, environmentally relevant data.

under constant threat by the Heisenberg uncertainty principle (see Section 6.4). For this reason, the first sampling step in Figure 6.4 is labeled "capture". The idea is to *capture information about the field site's microbial community before the validity of the information drifts away*. Pragmatically, the capture step is the initial physical contact between the investigator and the water, sediment, or soil of interest.

- **How do you remove a sample from the field? Is it physically disturbed?**
- **If so, how much time passes between disturbance and stabilizing the information delivered by the final analysis?**

As shown in Figure 6.4, the capture step may involve initiating a field incubation by placing a chamber over the field site so that dynamic changes in headspace gases (e.g., methane, CO_2, N_2O; see Sections 6.8, 7.3, and 7.4) can be monitored. If a field chamber is installed, the investigator must complete the assays rapidly so that the chamber, itself, does not alter microbial activity due to physical disturbances, restriction of gaseous exchange, and/or buildup of greenhouse heat. For static, snapshot-type assays aimed at describing community composition via its biomarkers,

viability of cells is not of concern; thus, fixation of cells and their biomarkers in a field-like state should occur immediately after capture (Figures 6.4 and 6.5). Assays that involve incubation of environmental samples so that dynamic physiological measurements can be completed (e.g., sulfate reduction, methanogenesis, biodegradation of environmental pollutants) require viable cells; therefore, fixed samples cannot be used. Under these circumstances, an investigator's best strategy is to complete the dynamic physiological assays as rapidly as possible, while taking steps to mimic in situ conditions.

The "store" step in Figure 6.4 recognizes that time passes between capture of the sample and completing the analytical procedures that produce information about the microbial community. If samples are fixed in the field (bound for geochemical, biomarker, or microscopic assays) they must be stored so they do not change. If samples are bound for physiological assays (Figure 6.5), the most widely recommended sample-holding procedure is cooling of the samples on ice until laboratory processing. The microbial populations present in soil and water samples may begin to shift and change the moment an environmental sample is removed from the field site (see Sections 6.2 and 6.4). These changes continue through cold storage, distribution of the samples, assay vessels, and continued laboratory incubation during the assays. It is this inevitable, intractable set of microbiological changes (as well as our inability to match laboratory to in situ field conditions) that often make it unwise to extrapolate the results of laboratory physiology assays directly to field sites.

Sampling for habitat characterization

The capture–fix–store–analyze scheme of Figure 6.4 applies equally well to samples from field sites aimed at habitat characterization. To develop hypotheses about field selective pressures, resources exploited by microorganisms, electron donor–electron acceptor relationships, and biogeochemical processes catalyzed by microorganisms (see Sections 3.7, 3.8, and 7.4), we must have accurate data describing in situ field geochemistry. To obtain field geochemical databases, individuals conducting site surveys must be sure that results of analyses accurately reflect true field conditions, not postsampling changes in the samples. *Thus, there is a critical need to relate results of geochemical measurements, performed on field samples, directly to processes and conditions in the field*. Therefore, the utmost care must be taken to avoid artifacts that may be imposed on analytical results by imprudent delays in analysis completion, improper sample fixation, or laboratory incubations. Whenever possible, portable field instruments should be used. Needs of deep sea marine-exploration research have led to significant technological advancements in portable mass spectrometers that can be deployed on submersible vehicles (e.g., Camilli and Duryea, 2009; Camilli et al., 2010;

Table 6.2). In subsurface habitats, cone penetrometry (Chiang et al., 1992; Robbat et al., 2010) and a variety of in situ water and off-gas analyses (Patterson et al., 2013) and sample gathering and fixation procedures are relevant to understanding on-site biogeochemical processes. As indicated in Table 6.2, however, many measurements cannot be completed in the field. When sample removal from field sites cannot be avoided, a variety of crucial decisions must be made. Selecting the locations within field sites for obtaining representative samples is no simple matter (Parkin and Robinson, 1994; Wollum, 1994).

Table 6.2

Organic and inorganic chemical assays pertinent to understanding the biogeochemistry of field sites. (Modified from Madsen, E.L. 1998b. Theoretical and applied aspects of bioremediation: the influence of microbiological processes on organic compounds in field sites. In: R. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (eds), *Techniques in Microbial Ecology*, pp. 354–407. Oxford University Press, New York. By permission of Oxford University Press)

Analytical approach	Sample preparation	Information	References
Portable field meters	None is required if probes can be immersed in soil, sediment, or waters in situ	Temperature, O_2, conductivity, and other measures pending availability of specific-ion electrodes and other analytical probes and standards	APHA (2012) and a variety of commercial manufacturers (e.g., Yellow Springs Instruments, Inc., Yellow Springs, OH)
In situ mass spectrometry	None is required if probes can be immersed into the habitat of ineterest	Dissolved gases, organic compounds	Camilli,and Duryea, 2009; Lebedev, 2013; Camilli et al., 2010
Instruments in mobile field laboratories (e.g., spectrophotometer, GC, and others) and in base analytical laboratories (e.g., generally higher precision spectrophotometer, GC, GC/MS, HPLC, HPLC/MS*, ion chromatography, and others)	Sampling, gathering, and fixation protocols vary with each specific assay. Fixation is designed to avoid chemical artifacts that may develop between the time that samples are removed from the field site and assays are completed	Inorganic nutrients and both electron donors and electron acceptors (e.g., O_2, NH_3^- Fe^{3+}, Fe^{2+}, Mn^{4+}, Mn^{2+}, NH_3^+ S^{2-}, SO_4^{2-} H_2, CO_2); organic constituents (e.g., the contaminants, co-contaminants, metabolites, dissolved and total organic carbon)	US Environmental Protection Agency methods as outlined by Christian (2003), Fifield and Haines (2000), Dane and Top (2012), Sparks et al. (1996), Dick (2011), Bottomly et al. (1994), Wagner and Yogis (1992), Keith (1996), and a variety of commercial manufacturers (e.g., Hach Inc., Loveland, CO)

*GC, gas chromatography; HPLC, high performance liquid chromatograph; MS, mass spectrometry.

Site heterogeneity and field analysis of electron-accepting processes

- **Should each sample be considered unique and treated independently?**
- **Or should samples be pooled (averaged) prior to performing measurements?**

Answer: Answers to such questions reside in the experimental goals and the methodologies employed (see below).

Deciding how to gather samples (with a shovel, spoon, pump, or drilling rig with or without aseptic techniques) can be a critical issue. Composite soil samples that contend with spatial heterogeneity are often utilized (van Wesemael et al., 2011; Corwin et al., 2010), as are other spatial and temporal statistical analyses (Yates and Warrick, 2012). In addition, the vessels for containing environmental samples must be clean, leakproof, and compatible with all intended uses. Once a field sample has been transferred to a container, the sample fixation protocol must be selected carefully to avoid artifacts. Obviously, fixation must prevent the parameter(s) of interest from changing and allows completion of the intended measurement procedures. Fixation may be accomplished by freezing (in liquid nitrogen or by placing samples on dry ice) or by adding biological inhibitors (e.g., formalin) and chemical fixatives (e.g., acid) (American Public Health Association (APHA), 2012).

Final electron acceptors that dominate the physiological reactions of field sites, or discrete zones therein, provide useful criteria for categorizing biogeochemical regimes (see Sections 3.7 and 3.8). Understanding these ambient conditions that control and respond to in situ physiological processes is essential. Site-specific efforts are required for defining which of the many possible biogeochemical regimes – for example, aerobic, denitrifying, iron-reducing, manganese-reducing, fermentative, dehalogenating, sulfate-reducing, or methanogenic (Morrice et al., 2000; Liou et al., 2010; Zehnder and Stumm, 1988; Morel and Hering, 1993; Hemond and Fechner, 1994; Lovley et al., 1994; Stumm and Morgan, 1996) – actually occur in the field. And, of course, spatial and temporal heterogeneity are the key impediments for successful site characterization. For example, in uniform, well-mixed aquatic habitats, consistent readings from an oxygen probe at a variety of locations and times can be interpreted accurately and extrapolated to the site as a whole. However, in soils and saturated sediments that are spatially heterogeneous, it is very difficult to know precisely where and when particular physiological regimes are established. All site characterization data must be interpreted in terms of the physiological processes that produce and consume geochemical constituents (Smith and Harris, 2007). Key insights into in situ microbial physiology can be provided by field measurements of co-reactants and endproducts of microbial metabolism (e.g., CO_2, Fe^{2+}, Mn^{2+}, S^{2-}, NO_2^-, N_2O, NH_4^+, organic acids, methane, and

Table 6.3
Relationships between the in situ partial pressure of hydrogen gas in anaerobic field sites and the dominant terminal electron-accepting process at that site. (Compiled from Chapelle et al., 1997)

Hydrogen gas partial pressure (nm)	Dominant terminal electron-accepting process
<0.1	Nitrate reduction
0.2–0.8	Iron reduction
1–4	Sulfate reduction
5–30	CO_2 reduction – methanogenesis

other compounds indicative of electron-accepting processes; see Section 7.3), as well as by concentration gradients of final electron acceptors themselves (e.g., O_2, NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-}, see Sections 7.4 and 7.5) along site transects. In this regard, Chapelle et al. (1997) have devised a gas-sampling bulb protocol for anaerobic groundwaters in the field that, in combination with hydrogen gas determinations and Winkler titrations for oxygen (APHA, 2012), provides definitive information on dominant anaerobic redox couples (Table 6.3).

In interpreting field measurements, one must be mindful of the presence of microenvironments, which may allow localized pockets of anaerobiosis to occur in seemingly aerobic habitats. Furthermore, many of the reduced endproducts may diffuse away or be transported from the location where they were produced. For instance, detection of methane in field samples (from a natural gas-free locality) indicates that the highly reducing biogeochemical conditions associated with methanogenesis are operative in the vicinity of the sampling point. However, because methane is a volatile and mobile gas, its detection does not necessarily define the physiological activities in progress at the time and the location of sample removal.

6.6 BLENDING AND BALANCING DISCIPLINES FROM FIELD GEOCHEMISTRY TO PURE CULTURES

Assembling mass balances for geochemical components in field sites, distinguishing microbiological from other processes, and tracing circuitous routes of geochemical materials through food chains and oxidation/reduction reactions are formidable tasks (see Box 6.1 and Figure 6.2; see also Figures 1.3 and 1.4). Many environmental microbiologists have confronted this situation and concluded that such adversities are nearly insurmountable in efforts aimed at discerning what microorganisms are doing in field sites (Bull, 1980; Hobbie, 1993; Hobbie and Ford, 1993; Madsen, 1996).

The common way to contend with uncertainties of microbial activities is to initiate flask assays in the laboratory that monitor the chemical transformation(s) of interest in samples gathered from field sites. These laboratory assays provide definitive qualitative evidence for *potential* microbial metabolic reactions because sterilized or poisoned treatments can be examined as abiotic controls and mass balances are made possible by performing the assays in sealed vessels. For example, in the 1870s, while examining microbial transformations of nitrogen, Schloesing and Muntz (cited in Waksman, 1927) described a key link in the nitrogen cycle, nitrification (see Sections 7.3 and 7.4), by reporting that nitrate was formed from ammonia in nonsterile, but not in poisoned, columns of sand infiltrated by sewage effluent. However, as described in Sections 6.2 and 6.4, it is critical to acknowledge that measurements performed on laboratory-incubated environmental samples *reveal what may be, but not necessarily what is*, actually occurring in field sites.

Controlled model laboratory experiments allow a logical, reductionistic progression to proceed from field sample, to laboratory incubation, to enrichment cultures, to the isolation of pure cultures, and to elucidation of cellular and subcellular processes (Figure 6.6). This progression is the source of information presently available on ecological, physiological, biochemical, genetic, and molecular aspects of microbially mediated geochemical reactions. These model system approaches are powerful because of the control attained in the laboratory and the use of experimental designs that can address specific hypotheses. Ironically, this reductionism is another basis for environmental microbiology's Heisenberg uncertainty principle. As each layer of reductionism unfolds, the complexity of the experimental system under scrutiny diminishes (Figure 6.6). However, with each simplification step, the likelihood of the resultant information being ecologically relevant also diminishes. Perhaps the riskiest step in attempting to gain a mechanistic understanding of biogeochemical processes is the selection of pure cultures for study (see Section 6.2). With a growing number of exceptions such as disease-causing agents (see Section 6.11) or endosymbionts (Ruby, 1996, 1999; McFall-Ngai et al., 2013; Bright and Bulgheresi, 2010; see Section 8.1), whose ecological niche often allows them to act almost as pure cultures in nature, imperfect traditional methodologies and the complexity of field sites (see Section 6.3) have hampered environmental microbiologists' attempts to know which members of microbial communities are responsible for biogeochemical field processes (see Section 6.11). However, relatively recent convergence of a variety of new procedures (innovative cultivation approaches, high-throughput DNA sequencing, metatranscriptomics, metaproteomics, stable isotope probing, and fluorescence in situ hybridization microscopy (FISH)) have successfully revealed the identities and roles of a variety of *Bacteria* and *Archaea* in their native habitats (for example, *Geobacter* in the subsurface, *Polaromonas* in sediment, and *Prochlorococcus*, *Synechococcus*, *Pelagibacter*, *Alteromonas*, and *Nitrosopumilus* in the oceans; see Section 6.11).

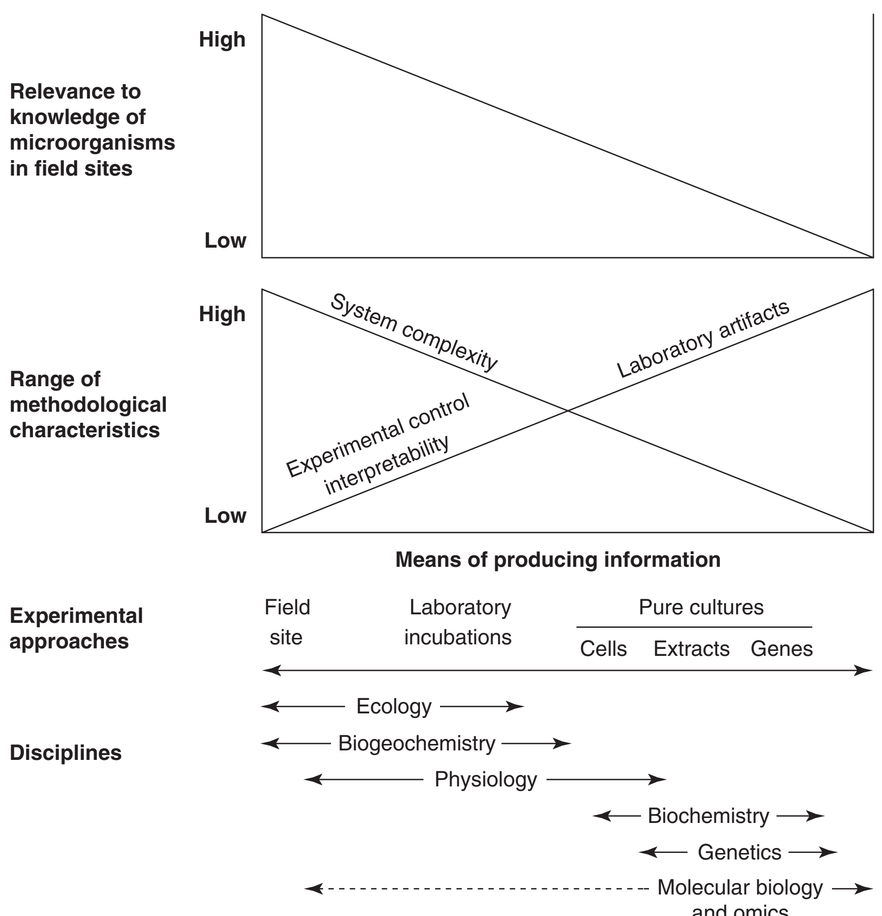


Figure 6.6 Relationships between means of producing information in environmental microbiology, their methodological characteristics, and their relevance to knowledge of microorganisms in field sites. As experimental approaches and corresponding disciplines become increasingly reductionistic (move from left to right), the relevance of the result information to microorganisms in field sites has traditionally diminished. (Reprinted and modified with permission from Madsen, E.L. 1998. Epistemology of environmental microbiology. *Environ. Sci. Technol*. **32**:429–439. Copyright 1998, American Chemical Society.)

The environmental relevance of data from pure-culture studies conducted in the laboratory is often suspect for at least two reasons:

1 The in situ biogeochemical process of interest is likely effected by intact naturally occurring microbial communities composed of complex mixtures of cells that often constitute intricate biochemical food webs (Schmitz et al., 2006; Sieber et al., 2012; Zinder, 1993; Schink and

Stams, 2006). Thus, the single organisms examined in pure-culture investigations are unlikely to be active or numerically dominant in nature and, therefore, may not be the correct objects of study.

2 Even if the pure-cultured organism being studied were responsible for the metabolic process in situ, the laboratory conditions used to grow and characterize the behavior of the organism may depart radically from the variety of influential in situ environmental factors (surfaces, colloids, gradients in substrate concentration, pH, final electron acceptors, etc.). The luxurious growth conditions sometimes provided in the laboratory may cause the metabolic process being studied to differ, quantitatively and perhaps qualitatively, from the process in situ where physical, chemical, and ecological constraints are likely to modify the organism's expression and regulation of genes (Lindow, 1995; Ramos et al., 2011; Damkiaer et al., 2013; Hibbing et al., 2010).

The void between field conditions in nature and physiological conditions in the laboratory is analogous to the void that separates cultured and non-cultured microorganisms (discussed in Section 5.1). Researchers on many fronts are actively working to better replicate natural selective and nutritional conditions that allow microorganisms to grow, to be isolated, and to carry out their normal biogeochemical processes in the laboratory (e.g., Button et al., 1993; Ward et al., 1995; Kaeberlein et al., 2002; Rappé et al., 2002; Könneke et al., 2005; Vartoukian et al., 2010; Nichols et al., 2010; Zengeler et al., 2007; Zengeler, 2008). These continued efforts will surely lead to increasing numbers of cultured microorganisms that are ecologically relevant (see Section 6.11).

In moving from left to right in the experimental approaches and scientific disciplines depicted in Figure 6.6, environmental microbiologists traverse from highly relevant but uncontrolled and sometimes uninterpretable field site measurements (see Figure 6.2 and Box 6.1) to sophisticated, yet simplified, experimental systems increasingly likely to induce artifacts and hence be of uncertain relevance to microbiological processes in nature. The nucleic acid-based surveys of microorganisms described in Chapter 5 (see Sections 5.2. 5.7, and 5.10) and Sections 6.7–6.11 often fail to detect microorganisms obtained via culture-based procedures (Ward et al., 1993; Pace, 1997; Janssen, 2006; Vartoukian et al., 2010; Nichols et al., 2010; Zengeler et al., 2007; Zengeler, 2008); thus, the free-living noninfectious, nonendosymbiotic microbial model systems studied in pure culture that supply virtually all of our knowledge of biochemistry, genetics, and molecular biology may not be ecologically significant. This does not mean that the fundamental biochemical and genetic processes revealed by laboratory-grown pure cultures have no bearing on ecological matters; indeed, many cellular processes, such as nucleic acid replication, ribosome structure, and adenosine triphosphate (ATP) generation are universal among virtually all life forms (Kluyver and van Niel, 1954; Neidhardt et al., 1990). Furthermore, laboratory experiments conducted on environmental samples, mixed cultures, and pure cultures have been invaluable in elucidating basic

physiological principles of methanogenesis, nitrification, denitrification, nitrogen fixation, sulfate reduction, and photosynthesis (among others) that control nutrient cycling in field sites (see below and Sections 7.4 and 7.6). However, biochemical divergence between field and laboratory metabolic processes should not be unexpected for many ecologically significant biogeochemical processes.

6.7 OVERVIEW OF METHODS FOR DETERMINING THE POSITION AND COMPOSITION OF MICROBIAL COMMUNITIES

Microorganisms removed from their native environments can be characterized microscopically (see Section 5.1). However, very little is known about the three-dimensional structure of microenvironments that surround microorganisms in field sites. New approaches such as environmental scanning electron microscopy and X-ray tomography are developing for examining complex environments such as soil. Yet complete microscopic characterization of soil is a distant possibility because the soil biomass occupies only 10^{-3} to 10^{-6}% of the soil volume (see Section 4.6). This means that a multitude of microscopic fields, each surveying a very small volume of soil, would need to be processed to obtain information accurately representing in situ spatial relationships of soil microorganisms. Even in relatively homogenous habitats, such as water columns in lakes and oceans, discerning dynamic, three-dimensional relationships within microbial communities has been elusive. Indeed, Stocker (2012) has recently made it clear that an emerging frontier in marine microbial ecology is understanding interactions between microbial behavior and the dynamic chemical and physical gradients in marine waters that establish "microarchitecture". Thus, unlike plants in landscapes (see Section 6.1), detailed knowledge of where microorganisms dwell is very difficult to obtain because of scale-related and sampling-related physical characteristics of microhabitats and microorganisms therein.

To answer the question "Who is there?", environmental microbiologists have developed four general types of assays already introduced in Box 5.1 and Section 5.10 and refined in Section 6.4 and Figure 6.5: (i) viable plate counts and isolation of organisms able to grow on laboratory-incubated selective agar media; (ii) extraction and analysis of nucleic acids, or other cellular biomarkers; (iii) microscopic examination of fixed, stained samples; and (iv) laboratory incubations that assess physiological potential of sampled microorganisms. Each of these methodologies has its own strengths and limitations. Common to the first three is the high probability of overlooking members of microbial communities that may be functionally significant but that may occur in low abundances and therefore be undetected. As we learned in Chapter 5 (Section 5.1), results of viable

plate count assays provide information about the small (<1%) proportion of the initially diverse mixture of microorganisms that are able to grow under physiological conditions imposed by limited resources presented to the microorganisms in laboratory-incubated media. Unmet challenges in designing the proper laboratory conditions for growing microorganisms are a major reason for such low cultivation efficiencies, but some microbial cells in natural samples may be damaged – rendering them moribund – difficult or impossible to resuscitate (see Section 5.1).

The microscopic approach for characterizing naturally occurring microorganisms typically disperses an environmental sample (e.g., soil), preserves it with a chemical fixative, and smears a portion onto a glass slide where the key microbial components (especially DNA, antigenic cell surfaces, or targeted nucleic acid sequences) can be stained (with general nucleic acid-binding dyes, cell-specific antibodies, or with gene-specific oligonucleotide hybridization probes (with or without signal amplification), respectively) to distinguish microbial cells from the inorganic and noncellular organic materials (see Section 5.1). General nucleic acid staining provides information on total microorganisms but usually falls short of providing information about the identity of individual cells because few types of microorganisms are morphologically distinctive. Microscopy can be combined with cell-specific (antibody and nucleic acid) procedures designed to allow particular microorganisms or metabolic processes (as expressed genes) to be recognized. The resulting assays can yield powerful insights into the composition and activity of naturally occurring microbial communities. Microscopic and other assays for process-specific biomarkers are discussed further in Section 6.11. When such microscopy-based probes are used, it remains a challenge to verify the specificity and accuracy of results from cells that probe positively in complex, naturally occurring communities. Of particular relevance to aquatic microbiology are flow cytometry procedures (e.g., Lomas et al., 2011) that interrogate (and sort) individual microbial cells as they pass, in single file, through the path of a laser beam. Information about cell-size distributions, the presence of photosynthetic pigments, and the binding of fluorophores that probe physiological state and biogeochemical function can be gathered from naturally occurring microbial populations (Lomas et al., 2011).

Extraction of cell-specific biomarkers has proven to be effective for some cellular components (such as phospholipids fatty acids; Tunlid and White, 1992; Findlay and Dobbs, 1993; Pinkart et al., 2002; Frostegard et al., 2011) but susceptible to inefficiencies and biases for others (such as nucleic acids; Moré et al., 1994; Farrelly et al., 1995; Suzuki and Giovannoni, 1996; Miller et al., 1999). Box 6.2 describes the use of phospholipid fatty acids in environmental microbiology. Historically, nucleic acid extraction followed by cloning and sequencing of phylogenetically revealing small-subunit genes (discussed below and in Sections 5.4 and 5.7) has provided evidence for novel bacterial, archael, and eukaryotic microbial residents of many habitats. When applied to a given field site, the results of this phenotype-free means of identifying

microorganisms usually contrast strikingly with those of growth-based assays. However, physiological inferences from phenotype-free methodologies can be misleading because prokaryotes that are closely related by small subunit rRNA sequence criteria can display widely different physiological and biogeochemical capabilities (Pace, 1997; see also Section 5.6).

Analysis of extracted nucleic acids, with emphasis on cloning and sequencing of small-subunit rRNA genes

Although revolutionary insights into naturally occurring microbial communities have been provided by nucleic acid approaches (see Section 5.7), they have their own methodological biases that shape the outcomes of

Box 6.2

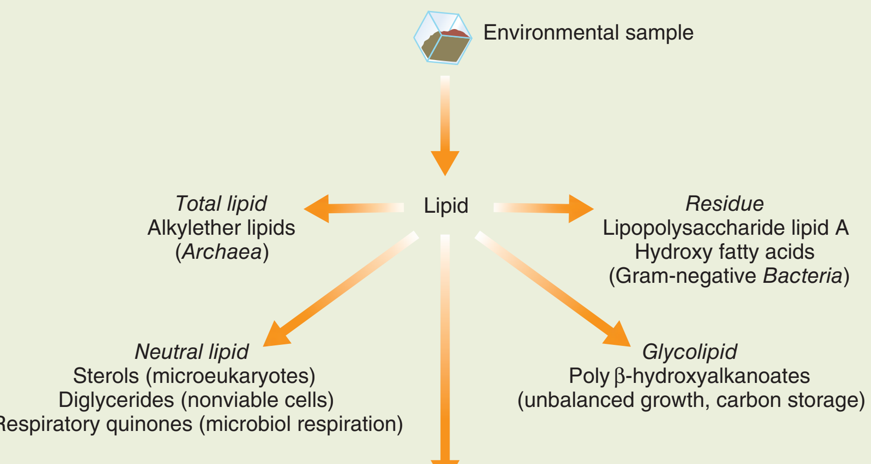
Using lipid biomarkers in environmental microbiology

A variety of non-nucleic acid biomarker molecules (e.g., phospholipid fatty acids, muramic acid, chitin, chlorophyll *a*) can be extracted from soil or other environmental samples to provide information about the abundance of specific groups of microorganisms (i.e., *Bacteria*, *Archaea*, fungi, algae). In utilizing these types of procedures, certain assumptions about the content of each biomarker per cell and their extraction efficiency must be carefully evaluated. Featured in this box are lipids, especially phospholipid fatty acids (PLFAs) because of the insights they provide into community composition. These insights are derived from the fact that various taxonomic microbial groups synthesize PLFAs of distinctive architecture. Structurally discernible characteristics of PLFAs (based on gas chromatography/mass spectrometry (GC/MS) analyses) include such traits as total number of carbon atoms per molecule, number of double bonds, position of the double bond relative to the omega end of the molecule, and whether or not the stereochemistry across the double bond is *cis* or *trans* (see below). The profiles of PLFA biomarkers from aquatic, sedimentary, and soil environments have been used to discern relative abundances of a variety of distinctive eukaryotic (e.g., plant, animal, microeukaryote) and prokaryotic (e.g., *Desulfobacter*, anaerobic desaturase pathways, *Bacillus*-type Gram-positive) groups (Tunlid and White, 1992; Findlay and Dobbs, 1993; Pinkart et al., 2002; Frostegard et al. 2011). The procedures used to obtain and interpret PLFA profiles are elaborate and perhaps best learned by direct instruction in laboratories where the techniques have been established.

Signature lipid biomarkers can be extracted from a variety of environmental samples and operationally divided into five pools (see below). The pool of greatest utility in environmental microbiology is ester-linked PLFA (of *Bacteria*). The ester-linked PLFAs are essential components of intact cell membranes that are unstable after cell death and lysis. Thus, when intact ester-linked PFLAs are extracted and analyzed, the resulting data describe the composition of viable biomass of *Bacteria*. There is a corresponding array of ether-linked lipid biomarkers that are characteristic of *Archaea* (Pearson and Ingalls, 2013; see Section 2.7).

Box 6.2 *Continued*

Signature lipid biomarkers



Intact polar lipids (phospholipid fatty acids, PLFAs)
Key cell membrane components of viable cells
(viable biomass, community composition, metabolic status)

Example

$$H{-}CH_2{-}CH_2{-}CH_2{-}CH{=}CH{-}CH_2{-}CH_2{-}(CH_2)_8{-}C({=}O){-}O{-}CH_3$$

Chemical structure of PLFAs

Glycerophospholipid (general structure)

$$^{1}CH_2{-}O{-}C({=}O)\sim\sim\sim$$ Saturated fatty acid (e.g., palmitic)

$$^{2}CH{-}O{-}C({=}O)\sim\sim{=}\sim\sim$$ Unsaturated fatty acid (e.g., oleic)

$$^{3}CH_2{-}O{-}P({=}O)(O^{-}){-}O{-}CH_2{-}CH_2{-}NH_2$$

Head group substituent: Ethanolamine

inquiries into microbial ecology (White, 1994; van Wintzingerode et al., 1997; Schloss et al., 2011; Temperton and Giovannoni, 2012). The basic approach to nucleic acid analysis of naturally occurring microbial communities is depicted schematically in Figure 6.7. "Environment" in Figure 6.7 is any habitat of interest supporting a naturally occurring microbial community. Every link in the chain of events from the top to bottom of Figure 6.7 must be carefully scrutinized and flawlessly implemented in order to obtain results that are truly indicative of the microorganisms native to the habitat. The environmental sample may need to be aseptically handled to avoid microbial contamination from irrelevant sources. Furthermore, whenever possible, the sample should be frozen immediately to avoid changes in the microbial community imposed by physiological perturbations during sample handling (see Sections 6.4 and 6.5). Nucleic acid

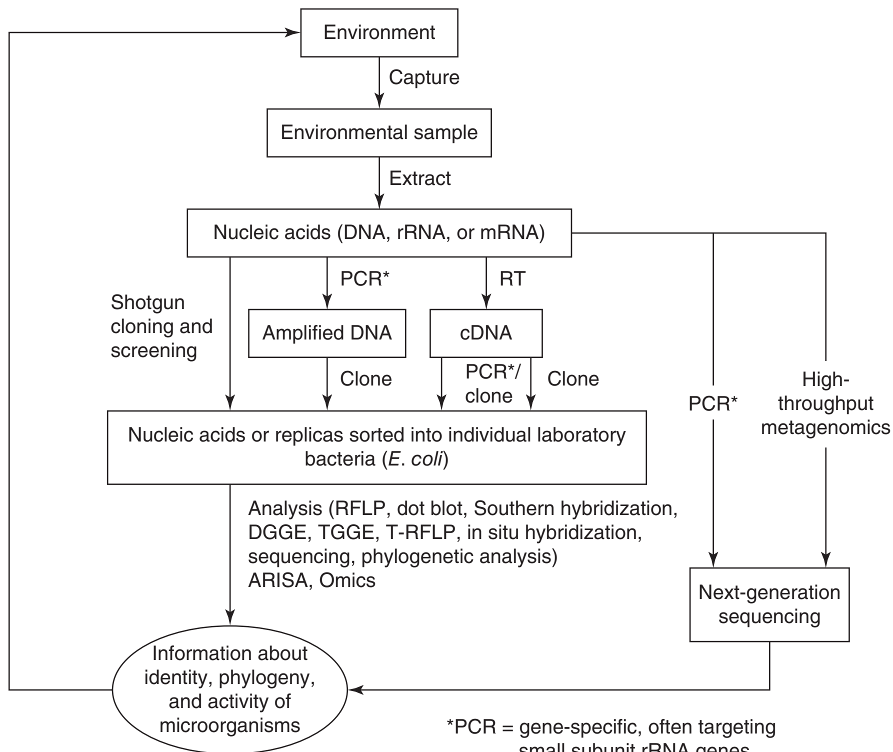


Figure 6.7 Stepwise scheme for carrying out nucleic acid analysis of naturally occurring microorganisms. For abbreviations, see Table 6.4. (Modified from Madsen, E.L. 2000. Nucleic acid procedures for characterizing the identity and activity of subsurface microorganisms. *Hydrogeol. J.* **8**: 112–125, fig. 1. With kind permission of Springer Science and Business Media.) Note that the high-throughput, next-generation sequencing procedures (right side) are discussed in Section 6.10.

extraction (Figure 6.7) is accomplished by a variety of physical and chemical procedures (Ogram, 1998; Miller et al., 1999; Petric et al., 2011; Griffiths et al., 2000) that lyse the cells and isolate the nucleic acids from accompanying cellular and environmental debris and solids (e.g., nonmicrobial biomass, detritus, sand, silt, clay, humic acids). The extraction step may be far less than 100% efficient and biased against lysis-resistant microorganisms (Moré et al., 1994).

After the nucleic acids have been extracted and purified, they exist as complex mixtures with individual molecular fragments (genes, partial genes, or sets of genes), often in low concentrations (Figure 6.7). Gaining information from the extracted nucleic acids requires that certain genes in the extracted DNA pool be sequenced and then analyzed bioinformatically. If knowing the taxonomic composition of the community is the goal, then the experimental procedures often focus exclusively on small-subunit rRNA genes. If knowing the total complement of genes in the community is the goal, then all genes are sequenced (this is metagenomics; see Sections 6.9 and 6.10). For both the small-subunit rRNA approach and the metagenomic approach, the sequencing may be preceded by a cloning step (Figure 6.7).

- Cloning-free, high-throughput next-generation sequencing approaches (focusing on both composition (16S rRNA) and metagenomics; right side of Figure 6.7) are discussed in Section 6.10.
- Cloning-based procedures for obtaining metagenomic information of microbial communities (focusing on all genes) are discussed in Section 6.9.
- Cloning-based procedures for obtaining information on the composition of microbial communities (focusing upon small-subunit rRNA genes) are described below.

The cloning process allows individual DNA fragments (with or without prior PCR amplification) to be incorporated into individual cloning vectors (such as those derived from plasmids or viruses; many of these cloning vectors have been designed by molecular biologists for genetic engineering purposes – see below and Section 6.9), which, in turn, are taken up by individual bacteria (e.g., *Escherichia coli*) that replicate in Petri dishes. The three basic ways to clone (obtain replicas of nucleic acids sorted into individual bacteria; Figure 6.7) are:

1. *Shotgun cloning*. This approach involves merging each individual extracted nucleic acid fragment with an individual plasmid or other vector, and allowing individual bacteria (e.g., *E. coli*) to take up the vector. This may be followed by screening of a large number of the recombinant bacteria (carrying a wide variety of nucleic acid fragments) for the rare gene(s) of interest.
2. *Polymerase chain reaction (PCR) amplification* from extracted DNA using primers for the gene of interest. These primers must be designed based on prior DNA sequence information that indicates highly conserved, specific regions of the gene that may flank variable regions of the gene;

such variable regions contain the nucleic acid sequence information of interest that reflects the identity or potential metabolic activity of microorganisms in the environmental sample.

3 *Reverse transcriptase (RT) step* that converts rRNA or mRNA to a complementary DNA (cDNA) template that can then be PCR amplified or directly cloned as described above (Amann et al., 1995; Liu and Stahl, 2002).

Thus, each bacterial colony that grows from the cloning steps shown in Figure 6.7 contains a single nucleic acid fragment originally present in the microbial community captured in the environmental sample.

None of the above three cloning strategies is artifact-free. All involve sequence-specific hybridization and binding for the amplification and merger (ligation) between vector and the sought nucleic acid fragment. Such molecular interactions are likely to favor some but not other sequences (Ward et al., 1995; van Wintzingerode et al., 1997). Sequence bias is likely to occur in all enzymatic processing (RT and PCR) of nucleic acids prior to cloning. Misrepresentation of the original community composition by PCR amplification and/or cloning has also been well documented (Farrelly et al., 1995; Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Frey et al., 2006) and is analogous to the shifts in before-and-after populations shown by the bar graphs in Figure 6.1. The physiologically active members of microbial communities are expected to have a high ribosome content (due to active protein synthesis), relative to dormant populations. Thus, community characterization based on extracted rRNA (later converted to cDNA via reverse transcriptase) intentionally targets active (not dormant) cells (Figure 6.7). A report by Tanner et al. (1998) demonstrated a rarely anticipated threat to the validity of PCR-based cloning and sequencing procedures. These researchers discovered that rRNA sequences can be retrieved from reagent-only preparations to which no environmental nucleic acids were added. Thus, great care must be taken to avoid mistaking reagent-borne microorganisms for ones dwelling in the habitat of interest.

Subsequent to successful cloning of the nucleic acids of interest, various analytical procedures can yield the sought information describing the identity, phylogeny, and/or potential activity of the sampled microorganisms. Molecular biology offers many sophisticated tools for characterizing nucleic acids. Those listed near the bottom of Figure 6.7 (RFLP, dot blot, Southern hybridization, DGGE, TGGE, T-RFLP, ARISA, in situ hybridization, sequencing, and phylogenetic and/or other bioinformatic analyses) have been applied routinely in gathering information about the naturally occurring microorganisms that were the source of the nucleic acids (see Section 5.4). All of the above technical terms are explained in Table 6.4. Because of their significance, the community fingerprinting methods (T-RFLP, DGGE, TGGE, and ARISA) are described in Box 6.3. A key structural feature of the diagram in Figure 6.7 is the long vertical arrow that

Table 6.4

Glossary and explanation of molecular biological and other procedures and terms used to gain information about microorganisms by analyzing and manipulating nucleic acids. (From Ausubel et al., 1999; Alberts et al., 2002; Primrose and Twyman, 2006; and the Lyons website http://seqcore.brcf.med.umich.edu/doc/educ/dnapr/mbglossary/mbgloss.html)

Term	Meaning and use
16S rRNA	Small subunit RNA in ribosomes, whose sequence is the basis for molecular phylogeny and taxonomy of prokaryotes
454 pyrosequencing	(See Section 6.10.) The technology uses a massively parallel sequencing-by-synthesis (SBS) system capable of sequencing roughly 20 megabases of raw DNA sequence per 4.5 h run of the sequencing instrument. The system relies on fixing nebulized and modified DNA fragments to small DNA-capture beads in a water-in-oil emulsion. The DNA fixed to these beads is then amplified by PCR. Finally, each DNA-bound bead is placed into a small well on a fiber optic chip. A mix of enzymes is also packed into the well and the four nucleotides (TAGC) are washed in series over the chip. During the nucleotide flow, each of the hundreds of thousands of beads with millions of copies of DNA is sequenced in parallel. If a nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding one (or more) nucleotide(s). The addition of one or more nucleotides results in a reaction that generates a light signal that is recorded by the instrument. The patterns of emitted light are processed by a computer algorithm into the sequences of the original template. Prior to assembly, the length of each continuous strand of sequenced DNA is ~700 bp
ARISA	Automated ribosomal intergenic spacer analysis is a molecular fingerprinting procedure, related to T-RFLP, used to characterize microbial communities. ARISA relies on PCR to amplify the region of DNA that resides between 16S and 23S rRNA genes (for prokaryotes). Like T-RFLP, one of the PCR primers bears a fluorescence tag. However, because the intergenic region is highly variable in length (150–1200 bp), no restriction digest is required
BAC	Bacterial artificial chromosome: a cloning vector capable of receiving between 100 and 300 kb of target sequence. BACs are propagated as a minichromosome in a bacterial host. The size of the typical BAC is ideal for use as an intermediate in large-scale genome-sequencing projects. Entire genomes can be cloned into BAC libraries, and entire BAC clones can be shotgun-sequenced fairly rapidly
Biomarker probe	Fluorescently tagged molecule that binds specifically to biomarkers
Biomarkers	Cell components (e.g., membranes, cell walls, enzymes, nucleic acids) whose detection is evidence for the identity and/or activity of microorganisms
BLAST	Basic local alignment search tool: a computer program that identifies sequence similarities among genetic sequences. The BLAST family of programs compares and provides a similarity score for DNA or protein sequences after matching them to sequences in huge compilations of DNA or protein-sequence databases, such as Genbank and Swiss-Prot

Table 6.4 *Continued*

Term	Meaning and use
Blotting	A technique for detecting one RNA within a mixture of RNAs (a northern blot) or one type of DNA within a mixture of DNAs (a southern blot) or one type of protein within a mixture of proteins (a western blot). A blot can prove that one species of RNA or DNA or protein is present, how much there is, and its approximate size. Basically, blotting involves gel electrophoresis, transfer to a blotting membrane (typically nitrocellulose or activated nylon), and incubating with a radioactive probe. Exposing the membrane to X-ray film produces darkening at a spot correlating with the position of the DNA or RNA or protein of interest. The darker the spot, the more targeted molecule there was
cDNA	Complementary DNA: a piece of DNA copied from an RNA molecule (usually mRNA, but also rRNA) using the reverse transcriptase (RT) enzyme
Cosmid	A type of cloning vector used to clone large pieces (35–45 kb) of DNA. These are plasmids that have been modified to resemble virus particles (they have a "cos" site). Cosmids can be packaged into bacteriophage heads (a reaction that can be performed *in vitro*) and then efficiently introduced into bacteria
DGGE	Denaturing gradient gel electrophoresis: specialized 16S RNA gene PCR primers amplify a portion of the gene (~400 bp) and include a high "G + C clamp" that anchors one end of the double-stranded DNA to itself. The gene fragments are all the same size, but they move to different locations in a denaturing gradient gel because different sequences are immobilized (due to denaturation) at different locations in the gel. This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities. The result is a series of horizontal bands in a single lane of an electrophoresis gel. After separation, the separated individual bands (DNA fragments) can be isolated and sequenced
Dot blot	A procedure that distributes and fixes a variety of DNA standards in a matrix of distinct locations (as "dots") on to a nylon membrane. The dots are hybridized to unknown mixtures of labeled DNA. Locations where hybridization is strong reveals sequence similarity between known sequences and the unknown ones. Used to identify extracted or cloned DNA fragments, relative to known standards (see Blotting)
Emulsion PCR	Used in several next-generation sequencing technologies, Emulsion PCR isolates individual DNA molecules along with primer-coated beads in aqueous droplets in an oil phase. PCR then coats each bead with clonal copies of the DNA template, followed by immobilization for later sequencing
FISH	Fluorescent in situ hybridization: microscopic detection of cells whose biomarkers (e.g., DNA, rRNA) hybridize to fluorescently tagged probe molecules of known binding specificity
Flow cytometery	Analysis of biological material by detection of the light-absorbing or light-fluorescing properties of cells or subcellular fractions (i.e., chromosomes) passing single-file in a narrow stream through a laser beam. An absorbance or fluorescence profile of the sample is produced. Automated sorting devices, used to fractionate samples, sort successive droplets of the analyzed stream into different fractions depending on the fluorescence emitted by each droplet

Table 6.4 *Continued*

Term	Meaning and use
Fluorescent antibody	Antibody raised in the immune system of a rabbit (for example) to recognize a particular antigen, such as a protein specific to a cell or an enzyme involved in a cellular process. After purification, the antibody can be linked to a fluorescent marker so that the targeted cell or protein can be recognized in a sample of interest using fluorescence microcopy
Fosmid	A cloning vector. A fosmid is a type of cosmid (f-factor), which is like a plasmid, but is capable of containing much larger pieces of DNA, up to 50 kb compared to about 10 kb in a plasmid. Like plasmids, fosmids are circular. However, unlike plasmids, *E. coli* cannot carry multiple copies of a fosmid
Hybridization	The reaction by which the pairing of complementary strands of nucleic acid occurs. DNA is usually double stranded, and when the strands are separated they will rehybridize under the appropriate conditions. Hybrids can form between DNA–DNA, DNA–RNA, or RNA–RNA. The hybrids can form between a short strand and a long strand containing a region complementary to the short one. Imperfect hybrids can also form, but the more imperfect they are, the less stable they will be (and the less likely to form). To "anneal" two strands is the same as to "hybridize" them
Ion torrent, next-generation sequencing	See Section 6.10
Illumina sequencing	(See Section 6.10.) This massively parallel, sequencing-by-synthesis technology utilizes a reversible terminator-based method that enables detection of single bases as they are incorporated into growing DNA strands. A fluorescently-labeled terminator is imaged as each dNTP is added and cleaved to allow incorporation of the next base. Hundreds of millions of these single-nucleotide-addition events occur simultaneously in sequencing cells that support a two-dimensional array of DNA fragments derived from the sample of DNA being sequenced
Immunofluorescent probe	Microscopically visualized fluorescently tagged antibodies that recognize cell-specific antigens
In situ hybridization	See FISH
Mate-pair sequencing (next generation)	Similar to "paired-end sequencing", except here the physical distance between the reads from the double-stranded DNA template is very long (2 to 5 kb in length). This is a chromosome mapping tool that assists in sequence assembly. There is no chance for overlap of sequences from the two reads
Microarray	Microarrays, otherwise known as "genechips", are tools developed originally by eukaryotic biologists to survey gene expression. The mRNA pool from a given tissue or cell line is used to generate a labeled sample which is hybridized in parallel to as many as 8000 DNA sequences that are immobilized on to a solid surface in an ordered two-dimensional array. Microarray technology can reveal patterns of genes that are activated under experimentally manipulated conditions
Microautoradiography	Use of microscopy to visualize silver grains created in photoemulsions by radioactive substances incorporated into microbial cells

Table 6.4 *Continued*

Term	Meaning and use
mRNA	Messenger RNA: an RNA molecule, transcribed from DNA, that contains sequences translated by ribosomes into proteins
Multiple displacement amplification (MDA)	Trace amounts (femtogram) of DNA template are reportedly copied by a branching mechanism in which phi 29 DNA polymerase extends random hexamer primers while this enzyme's strand displacement activity concurrently displaces previously made copies. An average DNA strand length is 12 kb
Northern hybridization	Transfer of RNA to a solid membrane where, after fixation, it can be hybridized against a variety of labeled nucleic acid templates so that the sought RNA fragment(s) can be identified (see Blotting)
Omics	The family of disciplines (e.g., genomics, proteomics, transciptomics, metabolomics, etc.) that systematically compile, analyze, and interpret bioinformatics data
Paired-end sequencing (next generation)	A modification of standard, single-read DNA library preparation in which relatively short stretches of double-stranded DNA are read. By linking single reads from the complementary strands together (by overlap), the effective read length is extended by ~50%
PCR	Polymerase chain reaction: a technique for replicating a specific piece of DNA *in vitro*, even in the presence of excess nonspecific DNA. Primers are added (which initiate the copying of each strand) along with nucleotides and Taq polymerase. By cycling the temperature, the target DNA is repetitively denatured and copied. A single copy of the target DNA, even if mixed in with other undesirable DNA, can be amplified to obtain billions of replicates. PCR can be used to amplify RNA sequences if they are first converted to DNA via reverse transcriptase. This two-phase procedure is known as RT-PCR (see RT)
Phylogenetic analysis	A set of quantitative approaches (manifest as computer algorithms) that infer evolutionary relationships among DNA and protein sequences. Often multiple alignments are analyzed – allowing groups of related sequences ("clades") to be displayed in relation to their relatives or neighbors on evolutionary trees. Commonly used computer programs include PHYLIP and PAUP
Plasmid	Cloning vector and naturally occurring extrachromosomal circular DNA of bacteria. *E. coli*, the usual bacterium used in molecular genetics experiments, will replicate plasmid DNA, as long as the plasmids have an "origin of replication". Plasmids carry inserted (cloned) DNA and produce millions of copies of the cloned insert
Quantitative PCR (qPCR)	A technology that allows a user to estimate the initial number of DNA templates that were present at the beginning of any given PCR reaction. Procedures rely upon spectrophotometric monitoring of PCR reactions as they occur in real time within individual wells of microtiter plates. Specialized PCR primers generate fluorescent signals with each PCR cycle. Calibration curves allow the user to relate the initial number of target DNA sequences to the number of PCR cycles required to reach a selected threshold of intensity of fluorescence referred to as "Ct"

Table 6.4 *Continued*

Term	Meaning and use
Real-time PCR	See Quantitative PCR
REP-PCR (RAPD-PCR, ERIC-PCR)	Repetitive extragenic palindromic sequence-PCR. This is one of the several genetic finger printing assays that relies upon the binding of PCR primers to specific but unknown sites in a given genome. Other related procedures include random amplified polymorphic DNA-PCR and Enterobacterial repetitive intergenic consensus-PCR. These procedures generate gel electrophoresis banding patterns, often at very high resolution
RFLP	Restriction fragment length polymorphism: a pattern-generating procedure that uses gel electrophoresis to separate DNA fragments that result from the recognition and cutting of an initially continuous piece of DNA by one or more DNA-cutting (restriction) enzymes
RNAseq	Whole transcriptome sequencing, made possible by applying next-generation DNA sequencing technologies to cDNA derived from RNA pools originating in pure microbial cultures or in complex microbial communities. rRNA depletion usually precedes preparation of cDNA libraries
RT	Reverse transcriptase: an enzyme that will make a DNA copy of an RNA template. A DNA-dependent RNA polymerase. RT is used to make cDNA
Sequence	As a noun, "sequence" is the order of monomeric subunits in DNA or RNA or protein molecules. For DNA, the sequence is the arrangement of A, T, G, and C bases it contains. As a verb, "to sequence" is to determine the structure of a particular DNA, RNA, or protein molecule: for instance, the specific sequence of nucleotides in a piece of DNA
Shotgun sequencing	A way of determining the sequence of a large DNA fragment. The large fragment is broken into many small pieces (~3 kb), and then each is taken up by a plasmid in an *E. coli* host and sequenced. By finding out where the 3 kb pieces overlap, the sequence of the larger DNA fragment becomes apparent. Many regions of the original fragment will be sequenced several times; this overlap or "clone coverage" is necessary and allows assembly of the pieces
Single-cell genomic sequencing	The few femtograms of DNA in a single bacterium can be amplified to microgram quantities of high molecular-weight DNA suitable for both traditional Sanger- and next-generation sequencing. The DNA amplification step is known as MDA (multiple displacement amplification) accomplished by phi 29 DNA polymerase
SIP	Stable isotope probing: following compounds bearing stable isotopic atoms (signatures) into and through microbial communities to tag, separate, and, later, detect populations involved in metabolism of the compound
Stable isotopic signature	Distinctive ratio of heavy versus light atoms of a given element in a given compound; discernible by mass spectrometry
TGGE	Thermal gradient gel electrophoresis: analogous to DGGE, except that the denaturing gradient is determined by temperature instead of salts, which alter the binding strength of nucleic acid strands to one another. This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities

Table 6.4 *Continued*

Term	Meaning and use
Transposon	Transposons are one of three types of transposable elements that facilitate the mobilization of genes from one location to another within a given chromosome. Transposase enzymes facilitate insertion of transposon DNA in new locations. Transposon mutagenesis is a molecular technique that intentionally introduces mutations as a means of genetic analysis
T-RFLP	Terminal restriction fragment polymorphism: PCR amplification of 16S rRNA genes inserts a fluorescent tag on one end of each of the amplified genes. After digestion with restriction enzyme(s), a single fluorescent molecule is formed from each amplified gene and the size of each fragment is governed by the gene sequence and the particular restriction enzyme(s) used in cutting the DNA. This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities. The DNA analysis instrument detects the size and intensity of fragments – resulting in a chromatography-like fingerprint of the community
Western blot	A technique for analyzing mixtures of proteins to show the presence, size, and abundance of one particular type of protein. Similar to southern or northern blotting, except that a protein mixture is electrophoresed in an acrylamide gel, and the "probe" is an antibody that recognizes the protein of interest, followed by a radioactive secondary probe (see Blotting)

connects the information generated back to the environment. As has been elucidated by Amann et al. (1995) and Madsen (1998a, 2005), routine application of methods from laboratory experiments to habitats of interest and back again provides a means to refine information and to develop and test new hypotheses about microorganisms in nature (see Section 6.11).

Developments in genomic techniques, including large-scale nucleic acid sequencing and the use of microarrays for surveying microbial communities for gene content and expression, are discussed in Sections 6.9 and 6.10.

Summary

Despite substantial sophistication in many of the above procedures assessing "Who is there?" in naturally occurring microbial communities, a complete census has yet to be successfully accomplished in any environment (see Section 5.4). Furthermore, of the millions of species of

Box 6.3

16S rRNA community fingerprinting: DGGE, TGGE, T-RFLP, and ARISA

DGGE (denaturing gradient gel electrophoresis)

Specialized 16S RNA gene PCR primers amplify a portion of the gene (~400 base pairs (bp)) and include a high "G + C clamp" that anchors one end of the double-stranded DNA to itself. The gene fragments are all the same size, but they move to different locations in a denaturing gradient gel because different sequences are immobilized (via denaturation) at different locations in the gel.

This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities. The result is a series of horizontal bands in a single lane of an electrophoresis gel. After separation, the separated individual bands (DNA fragments) can be isolated and sequenced.

Figure 1 shows a photograph displaying a DGGE analysis of six different environmental samples, each containing distinctive microbial communities (Muyzer et al., 1993).

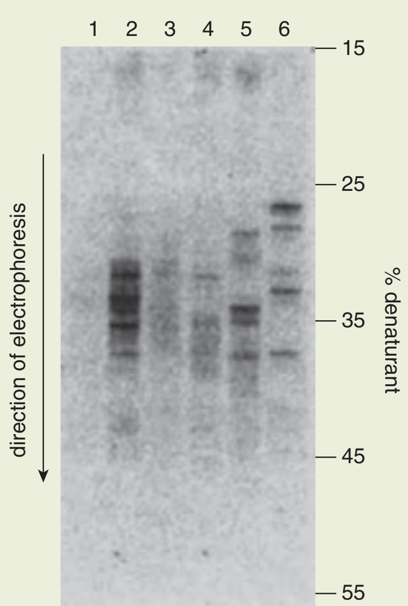


Figure 1 Denaturing gradient gel electrophoresis. (From Muyzer, G., E.C. DeWall, and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695–700. With permission from the American Society for Microbiology.)

TGGE (thermal gradient gel electrophoresis)

This is analogous to DGGE, except that the denaturing gradient is determined by temperature instead of salts that alter the binding strength of nucleic acid strands to one another.

This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities.

T-RFLP (terminal restriction fragment length polymorphism)

PCR amplification of 16S rRNA genes inserts a fluorescent tag on one end of each of the amplified genes. After digestion with restriction enzyme(s), a single fluorescent molecule is formed from each amplified gene and the size of each fragment is governed by the particular gene sequence and the restriction enzyme(s) used in cutting the DNA.

This is a DNA fingerprinting procedure that is applied to PCR-amplified 16S rRNA genes from microbial communities (Abdo et al., 2006). The DNA analysis instrument detects the size and intensity of fragments – resulting in a chromatography-like fingerprint of the community.

Fingerprints from four different environmental samples, each containing distinctive microbial communities, are shown in Figure 2 (Liu et al., 1997).

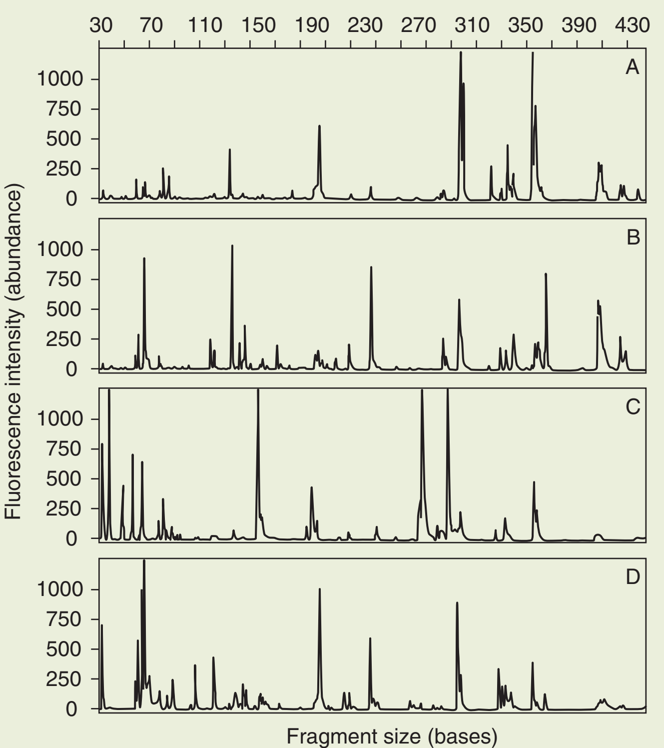


Figure 2 Terminal restriction fragment length polymorphism. (From Liu, W.-T., T.L. Marsh, H. Cheng, and I.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63**:4516–4522. With permission from the American Society for Microbiology.)

ARISA (automated rRNA intergenic spacer analysis)

This is a molecular fingerprinting procedure, related to T-RFLP, used to characterize microbial communities (Fisher and Triplett, 1999). ARISA relies on PCR to amplify the region of DNA that resides between 16S and 23S rRNA genes (for prokaryotes). ARISA has also been developed and applied in characterizing members of eukaryotic microbial communities such as fungi (bearing 18S rRNA). Like T-RFLP, one of the PCR primers is labeled with a fluorescent tag. However, because the intergenic region is highly variable in length (150–1200 bp), no digestion with restriction enzymes is required to develop and compare fingerprints of community composition. The length of the ARISA amplicon for a given bacterium is characteristic of that bacterium. A complex mixture of populations yields a multifragment ARISA pattern. A typical freshwater microbial community exhibits ~40 peaks in an electropherogram.

bacteria believed to exist globally, less than 14,000 have been characterized in traditional culture collections, and more than 100 times that number of rRNA genes from field-extracted nucleic acids have been sequenced. Thus, nucleic acid-based procedures provide a profound reminder that there is much knowledge yet to be gained about the microbial world. Additional information on biomarker-based assays of microorganisms and their potential metabolic activity is presented in Sections 6.9, 6.10, and 6.11.

Science and the citizen

Microbial source tracking to protect public health

Headline news: the United States Environmental Protection Agency (EPA) must monitor and protect water quality

In 1972, the Clean Water Act was passed by Congress and signed into law by the President of the United States (R.M. Nixon). The legislation set the goal of assuring that lakes, rivers, and streams in the United States remain "fishable and swimmable" (Simpson et al., 2002). Section 303(d) of the Clean Water Act requires that total maximum daily loads be established for undesirable materials that detract from water quality. Such materials include: (i) chemicals (e.g., fertilizers, pesticides, metals); (ii) physical parameters (e.g., turbidity, heat, discharged solids); and (iii) microorganisms (especially pathogenic bacteria, protozoa, or viruses). According to Simpson et al. (2002), approximately 35%, 45%, and 44% of the assessed rivers, lakes, and estuaries, respectively, have at times been classified as "impaired", based on concentrations of one or more of the three types of pollutants.

Effective management and protection of public health requires that waterborne microbial agents of disease be monitored and controlled. Clearly, in order to know the microbiological status of water, reliable and effective practices for detecting potential microbial pathogens must be established:

- What are the methodologies used in these practices?
- How do they contend with uncultured microorganisms?
- Do the practices help identify the sources of microbiological contamination so that the sources can be identified and curtailed?

SCIENCE: microbial source tracking is a continuously advancing discipline that uses both conventional (growth-based) and molecular tools to detect microbiological hazards and their sources (Santo Domingo and Sadowsky, 2007; Hagedorn et al. 2011; Roslev and Bukh, 2011)

Background

For many decades, public health authorities have relied upon fecal coliform bacteria (such as *E. coli*) as an index for the potential presence of potent pathogenic microorganisms commonly transmitted via feces. Coliforms (operationally defined as "facultatively" aerobic, Gram-negative, nonspore-forming, rod-shaped *Bacteria* that ferment lactose with gas formation with 48 h at 35 °C) are common inhabitants of the intestinal tracts of humans and animals. Because coliforms are not normal inhabitants of soils, sediments, or natural waters, coliforms are used as *indicators* of fecal contamination of water. Coliforms are particularly useful indicators because they can be recovered from environmental samples as culturable colonies that grow on specialized media where they can be readily classified and characterized. If detected, coliforms signal that additional detective work is appropriate to identify the source of the contamination and that pathogen-specific tests may be warranted.

Challenge
Detection of microbial pollution in a given water body tells us that pollution has occurred – but what we really need is a way to distinguish between different types of culturable coliforms and match them to source reservoirs.

Meeting the challenge with culture-based procedures
The culturable fecal microorganisms found in waters can be subcultured and their resistance to different antibiotics can be determined (see Section 8.7). The phenotypic trait of antibiotic resistance is largely habitat-based. Human-derived fecal bacteria have greater resistance to antibiotics used in human medicine, while animal-derived fecal bacteria show their own spectrum of resistances. In a given watershed, there are a finite number of potential sources of microbial contaminants (e.g., animal production facilities, septic systems, sewage outfalls). By surveying patterns of antibiotic resistance in the source reservoirs and matching them to those found in contaminated waters, the offending culprits can be identified and forced to improve their waste-management practices.

Meeting the challenge with molecular biology procedures
Microbial source tracking (MST) practitioners are fully aware that indicator coliforms, though useful, do not tell the whole story. The main drawbacks are: (i) the detected indicator organisms are, themselves, not pathogens and (ii) "uncultured" microorganisms (see Sections 5.1, 5.7, and 6.7) are overlooked.

As a first step toward refining current microbial monitoring efforts, molecular techniques have been successfully applied in characterizing cultured fecal coliforms and other microbial contaminants. The procedures that have been used include RFLP, PCR using primers that produce high-resolution fingerprints known as "rep-PCR", and T-RFLP (for explanations of these terms, see Section 6.7 and Table 6.4). Rather recently, both high-density microarray technology (nearly 60,000 16S rRNA sequences; Dubinsky et al., 2012) and bar-code-based next-generation sequencing of community 16S rRNA genes (Knights et al,.2011) have also been used to characterize and compare entire microbial communities. These methods offer a superior degree of discrimination power, compared to most other methods.

The ultimate advancement in MST would assemble a network of highly sensitive *nonculture*-based monitoring procedures that target the entire spectrum of potential water-borne microbial pathogens (from viruses to prokaryotes to protozoa). Table 1 below shows currently available candidate methodological procedures that may strengthen MST approaches (Simpson et al., 2002; Roslev and Bukh, 2011; Boehm et al., 2013). Initial applications of the procedures listed in the table will be restricted to scientific research. Under the Clean Water Act, thousands upon thousands of water samples are gathered and tested annually; thus, logistical and economic constraints will determine which, if any, of the procedures shown in the table will be routinely implemented on a broad scale.

Table 1

Comparison of molecular typing methods that may be considered for MST. (Modified from Simpson, J.M., J.W. Santo Domingo, and D.J. Reasoner. 2002. Microbial source tracking: state of the science. *Environ. Sci. Technol.* **36**:5279–5288. Copyright 2002, American Chemical Society; Roslev and Bukh, 2011)

Method	Description	Advantages	Disadvantages
RFLP	Electrophoretic analysis where DNA is detected with probes after southern blotting	Reproducible	Technically demanding
		Most strains typeable	Many probes needed to achieve adequate discrimination
Ribotyping	Southern hybridization of genomic DNA cut with restriction enzymes, probed with ribosomal sequences	Works with most strains	Slow
		Automated	Complex procedure Inconclusive results
Phage typing	Testing for susceptibility to different types of phages	Does not require electrophoresis	Need access to phage libraries
		High level of host specificity	Not all strains typeable Technically demanding Inclusive results
rep-PCR	PCR is used to amplify palindromic DNA sequences	Discriminatory	Cell culture required
	Couple with electrophoretic analysis	Does not require knowledge of genomic structure	Requires large database of isolates
		Reproducible	Variability increases as database increases
DGGE	Electrophoretic analysis of PCR products based on melting properties of the amplified DNA sequence	Works on isolates and total DNA community	Technically demanding
		Reproducible	Time-consuming
			Limited simultaneous processing
LH-PCR	Separates PCR products for host-specific genetic markers based upon length differences	Does not require culturing	Expensive equipment required
		Does not require database	Technically demanding

Table 1 *Continued*

Method	Description	Advantages	Disadvantages
T-RFLP	Uses restriction enzymes coupled with PCR in which only fragments containing a fluorescent tag are detected	Does not require culturing	Expensive equipment required
		Does not require database	Technically demanding
Phylochip	Microarray-targeting 59,959 16S rRNA sequences across *Bacteria* and *Archaea*	Extremely broad taxonomic survey of potential "indicator" microorganisms	Technically demanding (PCR-labeling of samples, use of microarray chip reader)
PFGE	DNA fingerprinting using rare cutting restriction enzymes coupled with electrophoretic analysis	High discrimination	Long assay time
		Works with most strains	Limited simultaneous processing
		Reproducible	
		Conclusive results	
AFLP	DNA fingerprinting using both rare and frequent cutting restriction enzymes coupled with PCR amplification	High discrimination	Technically demanding
		Works with most strains	Expensive equipment required
		Reproducible	
		Conclusive results	
		Automatable	
Bar-code based small subunit rRNA next-generation sequencing	Thorough effort to characterize entire community made possible by low sequencing costs	High discrimination state-of-the-art for census taking	Overwhelming amount of data likely to demand labor and computationally intensive analyses

Table abbreviations: RFLP, restriction fragment length polymorphism; rep-PCR repetitive palindromic polymerase C reaction; DGGE, denaturing gradient gel electrophoresis; LH-PCR, length heterogeneity PCR; T-RFLP, terminal RFLP; PFGE, pulsed field gel electrophoresis; AFLP, amplified fragment length polymorphism.

An extremely thorough methods-evaluation study (43 multiple source tracking technologies applied across 27 laboratories) has recently been completed (Boehm et al., 2013). The goal was to access reproducibility and reliability of molecular procedures that discriminate between fecal DNA signatures associated with humans, cows, ruminants, dogs, seagulls, pigs, horses, and sheep. Among the most sensitive and discriminating assays were those that targeted *Bacteroidetes* (dominant fecal inhabitants) characteristic of humans, ruminants, cows, dogs, and horses. These quantitative PCR-based assays used new information to develop new targets; for example, marker DNA for pigs and seagulls were, respectively, mitochondrial DNA and the bacterial genus *Catellicoccus*.

Research essay assignment

If you were head of the World Health Organization (WHO), or the Centers for Disease Control (CDC), or the US Environmental Protection Agency (EPA), you would be facing the enormous task of protecting the public against potential disease-causing microorganisms. Yet you would also be faced with limited financial budgets and limited personnel needed to implement programs. How would you set priorities for deciding which potential environmentally transmitted pathogenic agents should receive attention? List the criteria (up to 10) that you would use in prioritizing your agency's focus on environmental monitoring for disease prevention.

Now use the world wide web and the published scientific literature to find the criteria actually used by the WHO, the CDC, or the EPA. Prepare an essay comparing your criteria with theirs.

6.8 METHODS FOR DETERMINING IN SITU BIOGEOCHEMICAL ACTIVITIES AND WHEN THEY OCCUR

Of the millions of microorganisms found in each cubic centimeter of soil, sediment, and water, there are thousands of species, each with complex genomes conferring the potential to carry out a variety of biogeochemical processes (see Chapters 3, 5, and 7). Furthermore, many naturally occurring microorganisms exist as spores or other resting, dormant, or nonviable forms (see Section 3.5). Thus, unlike the clear link between the presence of higher plant and photosynthetic activity (see Section 6.1), the presence of microorganisms in environmental samples provides few clues about their specific physiological functions in situ.

The question "What are microorganisms doing?" can be subdivided into "What is the general physiological status of the cells?" and "What specific geochemical activities are the cells engaged in?" To assess the general physiological status of microorganisms in field sites, environmental microbiologists again rely on samples that are usually physically disturbed by removal from the field. In addition, similar to procedures

inquiring about the composition of microbial communities (Section 6.7), information about physiological status can be obtained from measurements conducted on laboratory-incubated samples, from biomarkers extracted from the samples and/or via microscopic techniques. Several key indicators of the physiological status of microbial cells are shown in Table 6.5. These kinds of assays are insightful, but each is limited in the information provided and each carries artifactual risks associated with the Heisenberg uncertainty principle (Karl, 1986, 1995; Madsen, 1996; see also Figure 6.5 and below).

Methods for inquiring into the specific in situ geochemical activities catalyzed by microorganisms seek to document the impact of microbial activities on the chemical composition of soils, sediments, waters, and atmosphere. For some microbial activities, the geochemical materials of interest or related microbial metabolites are volatile gases; hence, the underlying net microbial processes are measurable using field chambers placed over the surface of habitats being studied (Figure 6.8) (e.g., Yavitt et al., 1990; Conrad, 1996). Examples include fluxes of nitrous oxide (see Sections 7.2 to 7.4) released from wetlands or fluxes of atmospheric methane consumed by microorganisms at the soil surface. However, when neither the geochemical materials nor their metabolic products are volatile, documenting field metabolic processes requires more elaborate strategies that include physiologically guided chemical analysis of field samples such

Table 6.5

Biomarkers used to assess the physiological status of microorganisms in microbial communities. (From Staley and Konopka, 1985; Karl, 1986, 1993; Frostergard et al., 2011; Tunlid and White, 1992; Findlay and Dobbs, 1993; Madsen, 1996; Pinkart et al., 2002)

Biomarker	Information conveyed
Ribosome content	Indicative of protein synthesis activity
Intracellular energy reserves (poly-β–hydroxyalkanoates, ATP)	Overall energy charge and nutritional status
Proportions of *trans/cis* or cyclopropyl phospholipid fatty acids or electron transport carriers	These membrane components reflect nutritional status and starvation
Time-course measurements of cell elongation, uptake of physiological substrates, or the reduction of dyes indicative of respiratory activity	These assays, performed on laboratory-incubated environmental samples, provide an indication of metabolic activity ranging from incorporation of radioactive nucleotides (^{3}H-thymidine) to production of $^{14}CO_2$ from ^{14}C-labeled substrates.
Frequency of dividing cells	Microscopy-based assay indicating that cells are doubling, in situ, prior to habitat sampling

Figure 6.8 Field-chamber approach for determining in situ biogeochemical activity. Shown is a cluster of open-ended stainless steel cylinders inserted into the soil surface. After being capped with red rubber septa, the cylinders allow changes in the concentration of head-space gases to be monitored over time. The gas-sampling syringe (shown) delivers the sampled gas to a gas chromatograph for analysis. The chamber pictured here (and larger ones, sometimes fashioned from plexiglass) is typically installed temporarily over soil or water habitats. Large chambers may be fitted with an internal battery-operated fan to insure uniform mixing of gases (e.g., CO_2, N_2O, and CH_4). (From J. Yagi, Cornell University, with permission.)

as those shown in Table 6.6. The credibility of such biogeochemical activity measures varies on a case-by-case basis with the habitat studied, the means of procedural implementation, and the microbiological process of interest. Most are influenced by Heisenberg-type uncertainties discussed above (see Sections 6.4 and 6.5).

Accurate knowledge of temporal aspects of microbial activity in field sites, addressing the question, "When are the microorganisms active?", is difficult to obtain. If field samples are fixed the moment they are gathered, then information subsequently gleaned after analysis completion can be considered indicative of the status of the microbial community at the time of sampling (see Section 6.4 and Figure 6.4; Madsen, 1996). This real-time type characterization of microorganisms in field sites is implicit in most field-chamber (Figure 6.8; Conrad, 1996) and microelectrode (Glud et al., 1994) investigations. Data indicative of microbial metabolic actually in real-time also include transient, unstable biomarkers (such as metabolites, proteins, or mRNA-transcripts of actively expressed genes) characteristic of processes ranging from photosynthesis to nitrogen fixation to biodegradation of organic pollutants.

Traditionally, knowledge of when microorganisms carry out key biogeochemical reactions has been uncertain – inferred after the fact. Just as net photosynthesis in higher plants is inferred by the presence of plant biomass, microbial decay processes in steady-state ecosystems (e.g., salt marshes, forests, grasslands) can be inferred from the steady state itself (Heal and Harrison, 1990; Likens and Bormann, 1995). More recently, the scale of temporal and spatial resolution for assessing mechanistic biogeochemical intricacies of microbial processes has substantially improved with time-series investigations that systematically monitor the population structure and/or physiological activity of microbial communities (e.g., a marine water column) in real-time (e.g., Giovannoni and Vergin, 2012; Ducklow et al., 2009; Fuhrman et al., 2006; Karl et al., 2012). At

Table 6.6

Analytical chemistry procedures to assess the physiological processes carried out by microorganisms in field sites. (From Karl, 1986, 1995; Revsbech and Jørgensen, 1986; Levin et al., 1992; Pichard and Paul, 1993; Glud et al., 1994; Weaver et al., 1994; Hodson et al., 1995; Ogram et al., 1995; Madsen, 1996; Wilson and Madsen, 1996; Wilson et al., 1999; Grossman, 2002; Hurst et al., 2007)

Chemical assay	Information conveyed
Stable isotope ratios and fractionation patterns in naturally occurring compounds and in pools of environmental pollutants and their metabolic products	Signature ratios of stable isotopes can link pools of microbial substrates (e.g., carbon compounds) to their metabolic products (e.g., carbon dioxide or methane)
Release of stable, isotopically labeled materials	Imposed stable isotopic labels can be used to track the flow of pools of microbial substrates (e.g., $^{15}N\text{-}NH_4^+$) into their metabolic products (e.g., $^{15}N\text{-}NO_3^-$) when nitrification is being examined)
Isolating a portion of the habitat for hypothesis-driven manipulations	Chemical analyses performed on upstream and downstream samples can reveal processes that occur between sampling locations (e.g., metabolism of a groundwater pollutant such as toluene)
In situ microelectrode measurements of chemical gradients	Gradients of substances, especially electron donors and electron acceptors (e.g., oxygen or nitrate), are created by microbial respiratory processes
mRNAs (individual RT-PCR-based mRNA or high throughput metatranscriptomics) and/or enzymes (specific enzyme-activity assays or high-throughput metaproteomics) indicative of gene expression (e.g., fixation of carbon dioxide, nitrogen fixation, biodegradation of organic pollutants)	Measuring cellular precursors to biogeochemical processes support hypotheses that the processes are in progress in a field site. Results are convincing when experimental designs include data from adjacent, inactive control sites – as well as geochemical field data supporting the occurrence of particular metabolic processes
Conducting physiological assays indicative of the metabolic activity of interest on laboratory-incubated field samples (e.g., methanogenesis, denitrification, or biodegradation of environmental pollutants)	Short-term incubations of field samples exposed to hypothetical substrates can confirm metabolic processes – especially when no lag time is found between addition of the substrate and subsequent metabolism

RT-PCR, reverse transcriptase polymerase chain reaction.

the global scale, marine photosynthesis (both CO_2 fixation and oxygen production) can be estimated based on satellite assessment of dynamic chlorophyll blooms across the oceans. Remarkably, time-series measurements of whole-community transcriptomes have begun to emerge – these are accomplished via technological innovations that include remote robotic sampling devices that fix the mRNA pool in marine water samples

at short (~4 hour) time intervals for later collection, processing, and sequencing of extracted mRNA from expressed genes (Ottesen et al., 2013, 2014). Such approaches have the promise of yielding information of whole-community metabolism and population interactions over cyclic periods of light and darkness.

6.9 CLONING-BASED METAGENOMICS AND RELATED METHODS: PROCEDURES AND INSIGHTS

A major theme of this chapter (and, indeed, this entire book) is that environmental microbiology is a methods-limited discipline. When new types of measurements arise, the resulting novel information shapes the discipline's intellectual landscape and allows investigators to forge new frontiers. "Metagenomics" is a recent example.

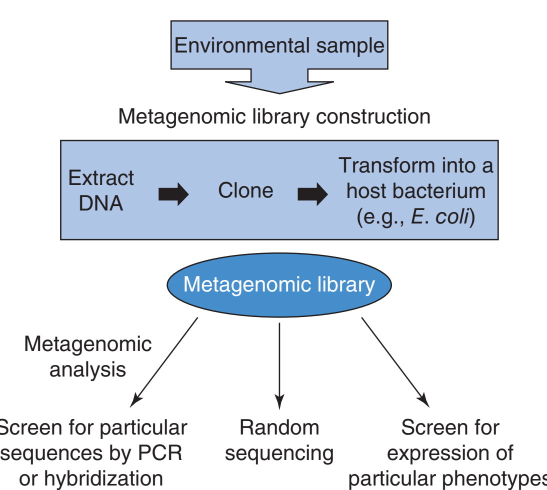


Figure 6.9 Overview of metagenomics: construction of a DNA library from mixed microbial populations in an environmental sample and three approaches for analyzing the sequences to reach conclusions about gene content. Both taxonomic (i.e., small subunit rRNA) and functional gene sequences are analyzed. The function of some genes can be confirmed by cloning and expressing them (as proteins) in bacterial hosts such as *E. coli*. PCR, polymerase chain reaction. (From Riesenfeld, C.S., D.D. Schloss, and J. Handelsman. 2004. Metagenomics: genomic analysis of microbial communities. *Annu. Rev. Genet*. **38**:525–552. Reprinted with permission from *Annual Review of Genetics*, Vol. 38. Copyright 2004 by Annual Reviews, www.annualreviews.org.)

Metagenomics

Metagenomics (also known as "environmental genomics" or "ecological genomics"; Handelsman, 2004; Allen and Banfield, 2005; Liu and Jansson, 2010; Gilbert and Dupont 2011; Hugenholtz and Tyson, 2008; Foster et al., 2012) is a methodology, sometimes creating vast data sets, that advances our understanding of biotic diversity and evolution. New hypotheses about the function of microorganisms and microbial communities can arise from metagenomic data. Metagenomics is a logical extension of biomarker analysis, already described within environmental microbiology's "tool box" (see Figure 6.5 and Box 5.1). Metagenomics applies the large-scale sequencing procedures (that have led to complete DNA blueprints of single organisms; see Section 3.2) to DNA from environmental samples that contain entire microbial communities. Note that *cloning-based metagenomics* are described in this section while *cloning-free metagenomics* are described in Section 6.10. Figures 6.9 and 6.10 provide a broad overview of the methods used in metagenomic studies. If you compare the

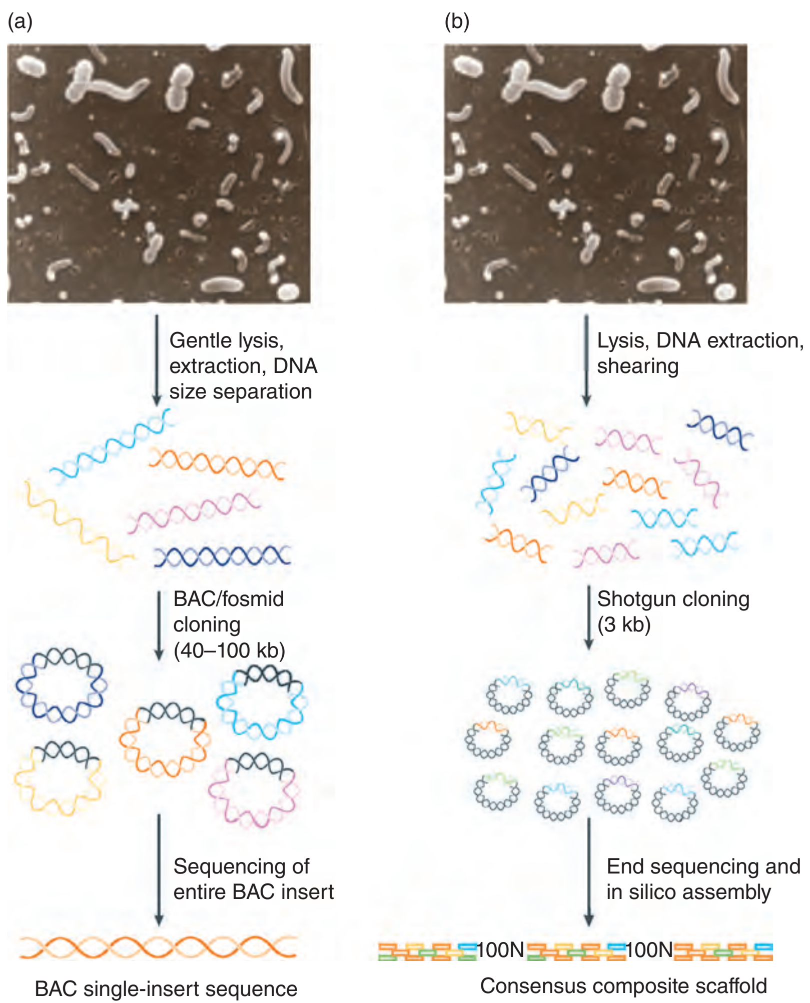


Figure 6.10 A glimpse of details of metagenomic methodology: microbial community DNA sequencing. Schematic diagram of common approaches for retrieving genomic sequence information from natural microbial populations. (a) One approach uses large DNA inserts recovered in bacterial artificial chromosomes (BACs) that are each derived from an individual cell. Subsequent sequencing and assembly results in a contiguous DNA sequence that is derived from a single cell in the original populations. (b) Another approach is based on recovery of small inserts, and attempts subsequent assembly from cloned DNA derived from a genetically heterogeneous population. The end result is an assembly of DNA sequence that is derived from many different cells. (Reprinted by permission from *Nature Reviews Microbiology*, from DeLong, E.F. 2005. Microbial community genomics in the ocean. *Nature Rev. Microbiol.* **3**:459–469. Copyright 2005, Macmillan Publishers Ltd, www.nature.com/reviews.)

nucleic acid-based community analysis already described in Figure 6.7 with Figures 6.9 and 6.10, the major contrasts are clear:

(i) In metagenomics there is an expansion of interest from small subunit rRNA (or other PCR-extracted genes) to all genes.

(ii) In metagenomics there is no PCR step that can distort the relative abundances of DNA fragments.

(iii) The size of the DNA fragment sorted into cloning vectors can be small (~3 kilobases (kb)) in the shotgun cloning approach or very large (~40–100 kb) in the bacterial artificial chromosome (BAC) and cosmid/fosmid cloning approaches.

(iv) The large (BAC or cosmid/fosmid) DNA fragments are subcloned, sequenced, and assembled into a single genomic fragment representing a portion of the genome from a single microorganism. Thus, associations between genes (especially 16S rRNA and functional genes) can be determined; these gene associations reflect those in a single uncultured host residing in the sampled community.

(v) The short (shotgun) DNA fragments are derived from genetically heterogeneous populations. Even with hundreds or thousands of 3 kb pieces of cloned DNA (that contribute to thorough "clone coverage"), the sequences from shotgun cloning may not be able to be assembled because the 3 kb sequencing lengths from complex communities may not share sufficient sequence similarity to be aligned to create coherent contiguous genomic fragments (contigs).

As shown in Figure 6.9, after the environmental DNA has been incorporated into a cloning vector and a bacterial host, there are three distinctive strategies for continuing the metagenomic analysis (Riesenfeld et al., 2004):

1. The random (shotgun) sequencing approach uses plasmids as vectors, and thousands of ~3 kb inserts represented in the clone library are sequenced. Early cloning-based "high throughput" sequencing projects (e.g., first three entries in Table 6.7) utilized instrumentation and facilities developed to sequence the human genome – especially the Sanger sequencer where read lengths were ~800 base pairs. The monetary expense of sequencing did not strongly influence project implementation – the investigators "do it all". It follows, then, that the shotgun approach to metagenomics generates respectably large DNA-sequencing data sets. Consistent with this, note that the clones obtained in entries 1–3 of Table 6.7 were not screened for particular genes of interest prior to sequencing; also, note that the numbers of base pairs sequenced is high (10^3 to 10^6 Kb of Sanger-sequenced DNA).
2. When a metagenomic investigation is focused upon retrieving large, intact DNA fragments (e.g., BAC cloning) derived from a single uncultured host bacterium, then the library of cloned DNA is normally screened prior to sequencing (Figure 6.9). If a known DNA sequence is being sought, then the screen for the clone library can be based on sequence-specific PCR or hybridization reactions performed on individual bacterial hosts carrying the cloned DNA. When the desired fragment is found, subcloning and sequencing is initiated (Figures 6.9 and 6.10). Entries 4–6 in Table 6.7 provide examples of metagenomic libraries prepared from various habitats (ocean water, insect endosymbionts, soil)

Table 6.7

Examples of cloning-based metagenomic investigations: their strategies, goals, and outcomes

Habitat sampled	Cloning strategy	Library screen	Vector	Size of inserted DNA (kb)	Total DNA sequenced (kb)	Outcome	References
1. Sargasso Sea (SS) and transit from SS through Panama Canal to Eastern Pacific Ocean	Shotgun	None	Plasmid	2.6	1.63×10^6 and 6.3×10^6	Mega-scale environmental genomics projects recovered ~1.2×10^6 genes (many novel) in the 2004 effort and 6.12×10^6 protein in 2007. Extremely useful database. Assembly of contigs from heterogenous populations is a challenge	Venter et al., 2004; Rusch et al., 2007
2. Acid mine drainage	Shotgun	None	Plasmid	3.2	7.6×10^4	Extremely simple microbial community was amenable to high clone coverage and assembly of sequences as two near-complete genomes and partial recovery of three others. Community metabolism was modeled	Tyson et al., 2004
3. Eastern Pacific Ocean	Shotgun	None	Plasmid		~7.4×10^2	Thousands of previously unknown, undiscovered virus sequences were found	Breitbart et al., 2002
4. Pacific Ocean	Large insert	16S rRNA	BAC	80	60	Novel *Archaea* and associated genes found in ocean waters	Béja et al., 2000
5. Beetle endosymbionts	Large insert	Antibiotic production	Cosmid	–	54	Novel pathway for synthesizing a polyketide antibiotics was discovered	Piel, 2002

Table 6.7 *Continued*

Habitat sampled	Cloning strategy	Library screen	Vector	Size of inserted DNA (kb)	Total DNA sequenced (kb)	Outcome	References
6. Soil	Large insert	16S rRNA	Fosmid	33–44	34	Novel link between uncultured soil *Archaea* (identified by the 16S rRNA genes) and associated genes	Quaiser et al., 2002
7. Soil	Large inset	Expression of antibiosis activity	Cosmid	–	~4	Discovery of genes that encode synthesis of antimicrobial compounds	Brady and Clardy, 2000
8. Soil	Small insert	Expression of lipase activity	Plasmid	2–7	613	Discovery of genes that encode lipolytic enzymes	Henne et al., 2000
9. Feces and soil	Large insert	Expression of biotin production	Cosmid	30–40	~11	Discovery of genes that confer biotin synthesis	Entcheva et al., 2001

BAC, bacterial artificial chromosome.

that led to the recovery of novel genes from uncultured hosts that shed light on microbial diversity (e.g., associations between hosts and their genes, or pathways of antibiotic production).

3 The third type of cloning-based metagenomic strategy shown in Figure 6.9 (entries 7–9 in Table 6.7) seeks new genes or gene clusters from clone libraries based on the encoded phenotype. Thus, the cloned genes are transferred to a host bacterium (e.g., *E. coli*, *Pseudomonas*, or *Streptomyces*). If the genes are expressed in the new host and the sought phenotype is detected, the cloned DNA is then sequenced and analyzed. This phenotype-based approach is generally aimed at discovering the genetic basis for commercially promising products such as antibiotics, nutritional supplements, or enzymes. The screening step in such investigations is crucial: if novel, potentially valuable, cloned genes are not expressed in the screening host, the genes will go undetected. A prominent series of cloning-based metagenomic investigations is summarized in Box 6.4.

Other genome-enabled procedures

Now that we have entered the "genomic and postgenomic" age in biology, there is a reasonably predictable progression in techniques used to advance our understanding of individual cultivated microorganisms:

1 The genome sequence provides a blueprint and rigorous basis for developing hypotheses about function, regulatory networks, and, ultimately, the behavior and ecology of the sequenced organism.

2 Hypotheses based on information yielded by the genome sequence can be tested using techniques that conduct comprehensive surveys of transcribed genes (i.e., the transcriptome assessed with microarrays (see below) prepared from the genome sequence and/or, more recently, RNA sequencing technology (RNA seq)), translated genes (i.e., the proteome, assessed with chromatography and mass spectrometry procedures that identify proteins extracted from cells using open reading frames (ORFs) in the genome as a guide for protein identification), and metabolites (i.e., the metabolome, the constellation of metabolites present in a cell at a given time, as assessed by chromatography and mass spectrometry).

3 Results from the "omics"-based procedures from point 2 are extended and confirmed using more traditional physiological and genetic (gene knockout and complementation) assays.

Part or all of the above three-step paradigm for advancing knowledge of individual organisms in pure culture has begun to be applied to intact, naturally occurring microbial communities. Three brief examples follow.

Example 1: Genomics of yet-to-be-cultivated microorganisms

Two major milestones for obtaining genomic sequences of uncultivated microorganisms have recently been achieved: (i) draft-genome assembly of individual microbial populations from high-throughput sequencing of community metagenomes (e.g., Albertsen et al., 2013; Wrighton et al., 2012; Hess et al., 2011; Denef and Banfield, 2012; see Section 6.10) and (ii) single-cell

Box 6.4

Metagenomics and the role of proteorhodopsin in the oceans: more than "chasing sequences"

One of the most remarkable scientific success stories arising from metagenomic investigations is the discovery of light-dependent proton pumps, known as proteorhodopsins, in microorganisms that occur throughout the oceans. The ongoing series of investigations have developed as follows.

Béja et al. (2000) discovered on a 140 kb BAC clone from the eastern Pacific Ocean (Monterey Bay) a new class of photosynthetic genes in the rhodopsin family (named proteorhodopsin). Also on the BAC clone was the 16S rRNA gene of an organism known as "SAR86". Taxonomically, the SAR86 bacterium was a gamma-proteobacterium known only as a 16S rRNA gene cloned from the Sargasso Sea (hence "SAR"). Nothing was known about SAR86 biology or associated genes until the BAC clone linked the proteorhodopsin gene to its host. When the proteorhodopsin gene was transferred into an *E. coli* host and expressed, the protein product of the gene actually functioned in *E. coli* as a light-driven proton pump. Prior to this report, rhodopsins had only been known to occur in extremely halophilic *Archaea*. *Thus, the function, the host, and the habitat for light-driven pumps (potentially able to pump protons, and hence contribute to cell metabolism such as ATP production) were all very exciting news. Metagenomics had possibly discovered an unknown and ecologically significant metabolic function*.

Béja et al. (2001) next reported from surveys of Monterey Bay waters, that both the genes encoding proteorhodopsin and the proteorhodopsin proteins, themselves, were widespread. To test whether proteorhodopsin-like molecules were functional in the planktonic microbial populations, the investigators analyzed membrane preparations from bacteria collected from surface water using a laser flash-photolysis technique (normally applied by biochemists to membrane preparations from pure cultures of photoactive organisms grown in the laboratory). The proteorhodopsin was there in cells native to the ocean. Moreover, new metagenomically produced BAC libraries (from Monterey Bay, the central Pacific, and the Southern Ocean) were found to contain proteorhodopsin-like genes. Sequence analysis of these proteorhodopsin genes showed structural variations in the encoded proteins. Furthermore, when expressed in *E. coli* the distinctive proteins exhibited distinctive differences in their light absorption spectra – one for shallow waters and one for deep waters.

Thus, the report by Béja et al. (2001) established the ubiquity of the novel proteorhodopsin gene in oceanic bacterioplankton, and suggested that the depth-dependent light intensities in the oceans had selected for specialized variants in the genes and proteins. *Overall, the global biogeochemical significance of proteorhodopsin-mediated phototrophy was becoming clearer: the hosts and proteins are widespread and the genes are stratified in depth-selected populations. Therefore, proteorhodopsin-based phototrophy is likely to have a significant impact on carbon and energy flux in the ocean*.

The next major installment in the proteorhodopsin story came from a series of initially independent investigations. 16S rRNA gene surveys had been used to discover another 16S rRNA gene sequence from the Sargasso Sea (deemed "SAR 11"; Giovannoni et al., 1990) that was later shown to constitute ~24% of the rRNA genes in major ocean waters. Rappé et al. (2002) were able to culture and isolate a bacterium representative of the SAR11 group of ubiquitous oceanic bacteria (strain HTCC1062; Figure 1).

The genome sequence of the recently cultured representative of Sargasso Sea-type ("SAR") bacteria, deemed *Pelagibacter ubique*, revealed proteorhodopsin genes (Giovannoni et al., 2005a). In a laboratory culture of *P. ubique*, the proteorhodopsin genes were expressed and the proteins exhibited absorption spectra similar to other previously characterized proteorhodopsins. However, when grown in filtered ocean water, *P. ubique* cells showed *no differences in growth rates or cell yields when in light or in darkness* (Giovannoni et al., 2005b). These investigations speculated that the phenotype conferred by proteorhodopsin genes may be subtle – important under special environmental conditions. This was prophetic.

The latest installments exploring proteorhodopsin ecophysiology have come from DeLong and Béja, (2010) and Steindler et al. (2011). Proteorhodopsin genes reside in many marine microorganisms (from *Pelagibacter* to *Vibrio* to *Salinobacter*). *Under conditions of starvation*, exposure to light allows cells expressing proteorhodopsin genes to show clear adaptive advantages that include enhanced survival, larger cell size, higher ATP content, and differential gene expression.

Summary

Béja et al. (2000, 2001) reported elegant experiments describing the biogeography of proteorhodopsin genes and their encoded proteins. While proteorhodopsin phenotypes had been explored by expressing the proteins in *E. coli*, the physiological role of proteorhodopsins in their ocean-derived host cells had not been confirmed.

Genome-enabled physiological studies were completed by Giovannoni et al. (2005b) on the SAR11 bacterium, which fortuitously was found to carry proteorhodopsin genes. These investigators showed that, despite being expressed, proteorhodopsin had no physiological impact. Later investigations (DeLong and Béja, 2010; Steindler et al., 2011) showed, that under specialized conditions of starvation, the photophysiology of proteorhodopsin is, indeed, physiologically and ecologically significant.

Lesson

Nonculture-based and cultured-based procedures go hand-in-hand. Early studies revealed the remarkable power of nonculture-based metagenomic inquiry. Hypotheses about previously unknown organisms and processes only arose from nonculture-based inquiry. However, to test the hypotheses, culture-based physiological (and other) assays are required.

Box 6.4 ***Continued***

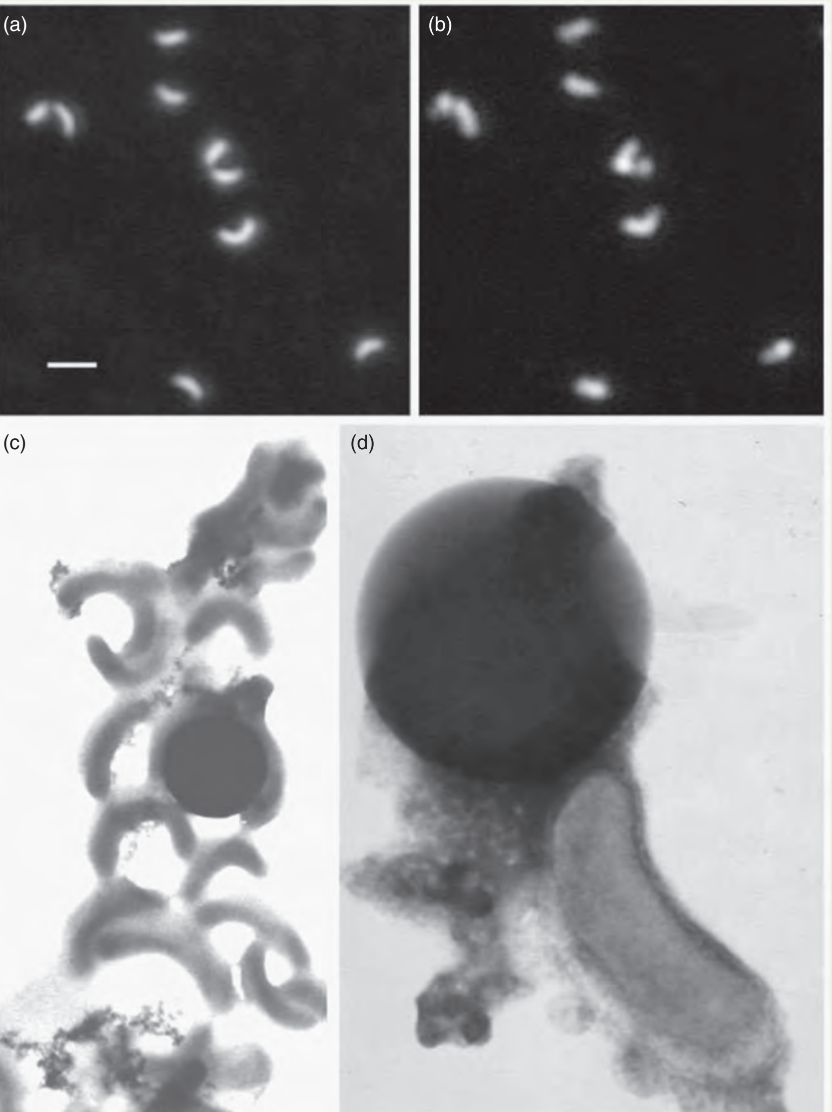


Figure 1 Photomicrographs of *Pelagibacter ubique*. (a), (b) Fluorescence images of cells in an identical field of view, stained with the DNA-specific dye DAPI (a) and after hybridization with four labelled oligonucleotide probes targeting SAR11 cells (b). Scale bar (a, b), 1 μm. (c), (d) Transmission electron micrographs of strain HTCC1062. (c) Shadowed cells with the typical SAR11 clade morphology. (d) Negatively stained cell. The latex beads in (c) and (d) have a diameter of 0.514 μm. (Reprinted by permission from Macmillan Publishers Ltd: *Nature*, from Rappé, M.S., S.A. Connon, K.L. Vergin, and S.J. Giovannoni. 2002. Cultivation of the ubiquitous SAR11 marine bacteriorplankton clade. *Nature* **418**:630–633. Copyright 2002).

genomics (Dean et al., 2001; Lasken, 2012; Marcy et al., 2007; Rinke et al., 2013, 2014). Because Section 6.10 discusses next-generation metagenomic methods and findings, only single-cell genomics will be discussed here. Flow cytometry (see Section 6.7 and Table 6.4) can lead to the isolation of single cells from environmental samples. Whole genome amplification (via multiple displacement amplification (MDA); see glossary, Table 6.4) can then be used to create μg quantities of DNA from the environment-derived single cell. This allows the sequencing and assembly of draft genomes (typically 40%, but sometimes 90% of the entire genome) that provide deep insights into the physiology, biochemical evolution, ecology, and phylogeny of the host of the sequenced genome. Furthermore, genomic clues about the organism's metabolism may lead to successful cultivation of the organism on a growth medium. When Rinke et al. (2013) applied single-cell genomic sequencing to samples of microbial communities from nine diverse environmental samples, these investigators were able to obtain (after screening for novel small subunit rRNA genes) 201 partial genomes from phylogenetically diverse *Bacteria* and *Archaea*. These genomes revealed previously unsuspected facts about rare mechanisms of gene transcription, horizontal gene transfer between domains of life, and helped to resolve uncertain lineages in the tree of life.

Example 2: Microarrays

Microarrays, otherwise known as "genechips", are tools developed originally by eukaryotic biologists to survey gene expression (Sharkey et al., 2004). The mRNA pool from a given tissue or cell line is used to generate a labeled sample that is hybridized in parallel to a large number of DNA sequences that are immobilized on to a solid surface in an ordered two-dimensional array. Commercially available microarrays are treated glass slides with >750,000 probes for genes (or "features") per array. Gene expression levels are normally presented as ratios of hybridization signals found at a given location (representing a given gene) in the array, for treated versus control cells (Sharkey et al., 2004). When applied to pools of mRNA, microarray technology can reveal patterns of genes that are activated under particular conditions (ranging from invasion of a human by a pathogen to denitrification by a soil bacterium), thus linking the activity of particular genes to processes catalyzed by their hosts.

In environmental microbiology, application of microarray technology is appealing because it has the potential to monitor changes in community composition [via 16S rRNA genes (Loy et al., 2002; DeSantis et al., 2003; Aw and Rose, 2012; Kellogg et al., 2012) or whole genomes (Wu et al., 2004; Bae et al., 2005)] and changes in functional genes associated with processes that cycle nutrients such as carbon, nitrogen, or sulfur (Dennis et al., 2003; Tarancher-Oldenburg et al., 2003; Zhou, 2003; He et al., 2012; Wu et al., 2006; see Chapter 7). Phylochip® technology (Kellogg et al., 2012) utilizes an array of ~60,000 16S rRNA genes for assessing microbial community composition, while GeoChip 4.0 contains ~ 84,000 50-mer oligonucleotides targeting 152,414 genes in categories of microbial functional (biogeochemical) processes. The degree of signal

intensity from the probed microbial community reflects experimentally chosen hybridization conditions and gene abundances relative to negative-control genes on the array. Like all promising techniques in environmental microbiology, microarray approaches must be applied prudently because they have strengths and weaknesses. Recent efforts suggest that improvements in microarray hybridization technology are ongoing: the composition of microbial communities have been successfully analyzed, while avoiding a potentially distorting (biasing) PCR step (DeAngelis et al., 2011).

Example 3: Phosphonates as P sources in the ocean

Phosphonates are a form of phosphorus in marine systems long known to chemists (see Table 7.4; McGrath et al., 2013), but the physiological impact of phosphonates was unknown until a clue was provided in the genome of the marine cyanobacterium, *Trichodesmium*. Under conditions of phosphate starvation, *Trichodesmium* was found to express genes and corresponding proteins involved in phosphonate uptake and metabolism (Dyhrman et al., 2006). Thus, genomics-based assays were essential in revealing a metabolic response by *Trichodesmium* to a previously unrecognized nutrient in ocean water. This discovery may explain *Trichodesmium*'s prevalence in low-phosphate marine waters and why microorganisms lacking the genes for phosphonate utilization may be restricted to high-phosphorous habitats.

Example 4: Creation of O_2 during anaerobic (nitrite-based) oxidation of methane

Anaerobic oxidation of methane is a crucial process that regulates atmospheric methane concentrations, and hence global warming and climate change (see Sections 3.11, 7.2, 7.3, and 7.4). History was made when Ettwig et al. (2010, 2012) applied genomic sequencing and both mRNA transcript and proteome analyses to an enriched consortium that oxidized methane anaerobically, while using nitrite as the electron acceptor. Shockingly, evidence was found showing that the canonical oxygen-dependent methane metabolism operated in this anaerobic culture. To explain how an oxygen-requiring physiological step can be supported by anaerobically grown cells, Ettwig et al. (2010, 2012) hypothesized, tested, and proved the existence of a previously unknown enzyme (nitric oxide dismutase) that converts 2 molecules of the intermediary metabolite NO to N_2 to O_2. The latter is then consumed intracellularly by a methane metabolic pathway initiated by O_2 requiring methane monooxygenase (see Section 7.5 and Box 7.10).

6.10 CLONING-FREE, NEXT-GENERATION SEQUENCING AND OMICS METHODS: PROCEDURES AND INSIGHTS

The goal of this section is to establish a foundation for understanding "omics" science and technologies and their impact on Environmental Microbiology. Much of the information presented here will be extended in

Chapter 9. Because omics technologies (genomics, transcriptomics, proteomics, metabolomics, etc.) are rapidly changing, some scientific details in this section may be dated. Nonetheless, it is hoped that the key principles and pivotal references presented here will be enduringly useful.

- **What are Omics Technologies and what types of information do they generate? (See Box 6.5 and below for an overview of next-generation DNA sequencing technologies. Box 6.6 provides a synopsis of bioinformatic analyses of environmental DNA sequences obtained using next-generation technologies.)**

According to Joyce and Palsson (2006), omics data sets are distinguished from other biological information in two ways: (i) their extremely broad scope – that holistically embraces the entire genome of one (or more) organism(s) and (ii) the depth of information, which is created by high-throughput technologies (such as DNA sequencing) that have propelled biology into a data-rich state, requiring computer-based information management known as bioinformatics. The "omics" suffix implies systematic and comparative examination of patterns in, and insights from, these large data sets: "genomics" for genomes, "transcriptomics" from transcriptomes, and "proteomics" for proteomes.

Box 6.5

Overview of DNA sequencing technologies: from Sanger to next generation (see also Table 6.8)

Sanger AB3730x1

In 1977, F. Sanger developed DNA sequencing procedures relying on a chain termination mechanism stemming from incorporation of dideoxynucleotides during synthesis of strands of DNA complementary to an unknown sequence. Applied Biosystems gradually built upon and improved Sanger's original technology by incorporating capillary electrophoresis and fluorescently labeled nucleotides. This constitutes "first generation" sequencing and set very high standards for both read length (up to 900 bp) and accuracy (99.999%) (Table 6.8). The drawbacks are low throughput, low total yield of sequence per run, and, correspondingly, high cost for high sequencing yields characteristic of the "next generation" technologies described below.

454 pyrosequencing

In 2005, the company named "454 Life Sciences" (later purchased by Roche), devised a sequencing technology relying on light-based detection of pyrophosphate released during nucleotide incorporation into growing DNA strands complementary to unknown DNA. Libraries of DNA fragments with 454 specific adapters are denatured to single strands, captured by 20 μm amplification beads, followed by an emulsion PCR step. This massively parallel "sequencing-by-synthesis" technology features millions of beads hosting the DNA libraries in each analytical run. Pyrophosphate released by nucleotide incorporation into growing strands leads to a luciferin-based burst of light, whose detection signals the sequence of template DNA. As shown in Table 6.8, key characteristics of 454 pyrosequencing are 700 bp read

Box 6.5 *Continued*

lengths and ~700 Mbp of total sequence per run. The advantages of 454 pyrosequencing include short time to completion and relatively long reads. Drawbacks include high cost of reagents, appreciable labor demands for each run, and a high error rate for strings of identical bases (polybases) longer than 6 bp.

Illumina

In 2006, Solexa (later purchased by Illumina) devised another massively parallel, "sequencing-by-synthesis", procedure that (after preparing a library of unknown template DNA) uses adaptor molecules to graft unknown single-stranded DNA fragments on to the surface of a flow cell. The fragments are amplified to form clusters of cloned templates and millions of these clusters are flooded with fluorescently labeled nucleotides, each of which releases a signal of fluorescent light when incorporated into a growing strand. As shown in Table 6.8, Illumina sequencing technology is currently available in three instruments with a range of costs per instrument, cost per run, sequencing yield, and read length. As of 2013, compared to 454 and SOLiD (see below), Illumina HiSeq 2500 was the least expensive technology per 10^9 bases. A drawback of Illumina sequencing technology is the long processing time (11 days) and the relatively short read length (~300 bp), but in a "paired-end" operating mode, this can be extended to ~600 bp.

PacBio SMRT

In the Pacific Biosciences "single molecule real time" sequencing approach, millions of 50 nm-wide wells (termed zero-mode waveguides) are the sites of attached individual DNA polymerase molecules. Each DNA polymerase carries out synthesis of a strand of DNA complementary to an unknown DNA fragment. As each sequential nucleotide is incorporated into a growing strand, a pulse of light (characteristic of each of the 4 nucleotides) is detected in real time. Positive traits of the PacBio technology are very short run times (2 h) and exceptionally long reads (as long as 10 Kb; average, 1500–3000 bp); corresponding negative traits include high capital cost for the instrument and a high error rate (~12%) (Table 6.8).

Ion Torrent

In 2010, semiconductor technology was devised to track the release of protons during DNA-polymerase-catalyzed incorporation of nucleotides into growing DNA strands. The semiconductor chip is consecutively flooded with four different nucleotides and changes in voltage reflect incorporation of particular nucleotides complementary to the unknown DNA strands being sequenced. Ion Torrent requires neither fluorescent signals nor camera-based scanning, resulting in a lower-cost instrumentation, higher processing speed, and lower-cost processing. Three different semi-conductor chips are available for Ion Torrent sequencing units (Table 6.8); these largely determine total sequence yield per run (from 20 Mb to 1Gb). The typical read length is 200 bp.

SOLiD

In 2006, sequencing by Oligo Ligation Detection (SOLiD) was acquired by Applied Biosystems. In a SOLiD flow cell, libraries of unknown DNA fragments are fixed into ~100 million 1-µm beads and then probed with known 8-bp sets of oligonucleotides. The process involves ligation of dinucleotides to the growing strand followed by both cleavage of the 5th base of the probe and flooding the cell with probes bearing dinucleotides linked to a distinctive fluorophore. Specificity of the di-base probe is achieved by interrogating every 1st and 2nd base in each

ligation reaction. After five rounds of sequencing using "ladder primer sets", the produced signals allow the sequence of template DNA fragments to be delivered. Large total sequencing output (120 Gb), long run times (7 days or 14 days), and very short read lengths (50 bp) are characteristic of SOLiD technology (Table 6.8).

Other emerging DNA sequencing technologies

Nanopore sequencing is under development. This technology relies upon preparation of single-strand DNA libraries whose constituent fragments are passed through a transmembrane channel formed by the haemolysin protein produced by *Staphylococcus aureus*. Electrical current is monitored during depolymerization of the DNA fragments and each nucleotide released is recognized by a characteristic alteration in ion current.

Full performance details were not available, though read lengths of > 5 kbp were suggested. The technology features a variety of appealing traits, including: the absence of reliance on fluorescently labeled reagents, no need for PCR processing of samples, and rapid processing time.

Polonator sequencing

This utilizes a ligation-based approach to record nucleotide incorporation into growing DNA strands. To sequence a genome, four tasks are completed: (i) preparation of a paired tag shotgun DNA library; (ii) amplification of the library on beads using emulsion PCR; and (iii) enrichment of the beads in a monolayer in a flow cell flooded with 9-bp probes bearing either of four fluorophores (one for each base). Imaging records sequential positions of ligated probes and decoding allows inference of the template DNA obtained from the original library.

Helicos technology

This uses a single-molecule sequencing approach. Unknown DNA is fragmented (100–200 bp) and billions of these fragments are adapted to be captured on to anchors in a flow cell. DNA polymerase sequentially adds fluorescently labeled nucleotides. Each batch of added nucleotides is imaged; then the fluorescent tags are removed so that the prior DNA strands can continue to be extended. The process cycles between sequential base addition and imaging. Throughput is billions of base pairs sequenced in a day or less.

Box 6.6

Bioinformatic analysis of environmental DNA sequences obtained using next-generation technology

When one's scientific goal is accurate sequencing of a single gene (e.g., the 16S rRNA gene (~1500 bp) in a single bacterial culture), the traditional approach has been simple and rigorous:

(i) Use Sanger sequencing technology to obtain three reads for the plus DNA strand and three reads for the minus DNA strand.

(ii) The three reads (#1, #2, #3) arise from sequencing primers that are designed to bind the template DNA at position ~1, ~500, and ~1000, respectively.

(iii) After quality-control inspection of sequencing traces and trimming of low-quality ends, ~800 bp reads are obtained.

Box 6.6 *Continued*

(iv) Overlaps in sequence reads #1 and #2 plus reads #2 and #3 allow contiguous strands (contigs) for both the plus and minus strands to be assembled.
(v) Complementarity of base pairing between plus and minus strands provides internal quality control and redundancy for the final gene sequence.
(vi) Six sequencing reads (and recognition of start site, stop site, promoters, and Shine–Delgarno ribosome binding sequences) have defined a gene.

Once this fundamental genetic unit, a gene sequence, has been obtained, then a variety of tools can be applied (basic local alignment search tool (BLAST), multiple gene alignments, phylogenetic inference, protein motif analysis, etc.) that place the gene in its biological context and allow hypotheses to be developed about the gene's function.

Next-generation sequencing technology scales up the above six DNA reads by a factor of 10^6 to 10^9! Clearly, bioinformatics-based data-handling systems are required (Figure 1; Prakash and Taylor, 2012). The millions to billions of raw sequencing reads generated in the first step shown in Figure 1 (the first arrow leading from "Environment" to "Metagenomic Reads" are made possible by next-generation sequencing technologies. However, it is the remaining steps shown in Figure 1 that convert the *sequencing data* into *biological information*. Reviews on bioinformatics analyses include those by Kunin et al. (2008), Prakash and Taylor (2012), Wooley et al. (2010,), Pignatelli and Moya (2011), and Yok and Rosen (2011). Algorithms prepared in computer code receive the output from next-generation sequencing devices and carry out the key steps outlined above (for the six Sanger reads) in an automated fashion: quality control, read denoising, sorting of the reads prior to assembly (often termed "binning"), assembly, gene prediction, and comparative analysis of the assembled sequences – including metabolic, phylogenetic, and genomic comparisons. Figure 1 shows the typical flow of information that occurs during bioinformatic analysis of environmental DNA sequences. One begins with nucleic-acid extracts from the microbial community dwelling in an environment of interest (top, Figure 1). After sequences are generated (first arrow at the top of Figure 1), there are about a dozen levels of processing that can be applied to the data (from the checking of read quality, to assembly, to gene prediction, to analyses of contigs, scaffolds, genes, gene clusters, draft genomes, and/or complete genomes). The upper half of Figure 1 shows how "Predicted Complete and Partial Gene Sequences" arise from the environment. For functional analysis of the predicted complete and partial gene sequences (lower half of Figure 1), there is no single "right" way to "know" the function of genes assembled from metagenomic reads. This is because all bioinformatics calls are estimates (recall from Section 3.2 the challenges of annotating individual genes during genomic analyses). The bottom half of Figure 1 shows four paths for "Functional Analysis" of the gene sequences. Three of the four strategies for functional sequence analysis are well defined: (i) "Homology-based" (far left of Figure 1) relies upon BLAST-type comparison of the sequence inherent in each assembled gene; (ii) "context-based" places an emphasis on gene neighborhoods, operon associations and/or regulation to infer functionality; and (iii) "motif and pattern-based" (far right of Figure 1) relies upon patterns in the structure of the active site of proteins translated from the gene sequences. The fourth strategy for gene annotation is "other functional analysis", which relies upon a variety of function-specific characteristics of particular enzyme types that range from carbohydrate-active enzymes to membrane proteins to transporters to adhesins to virulence factors (Prakash and Taylor, 2012). The five key categories of software tools that achieve gene functional analysis are as follows (associated computer program

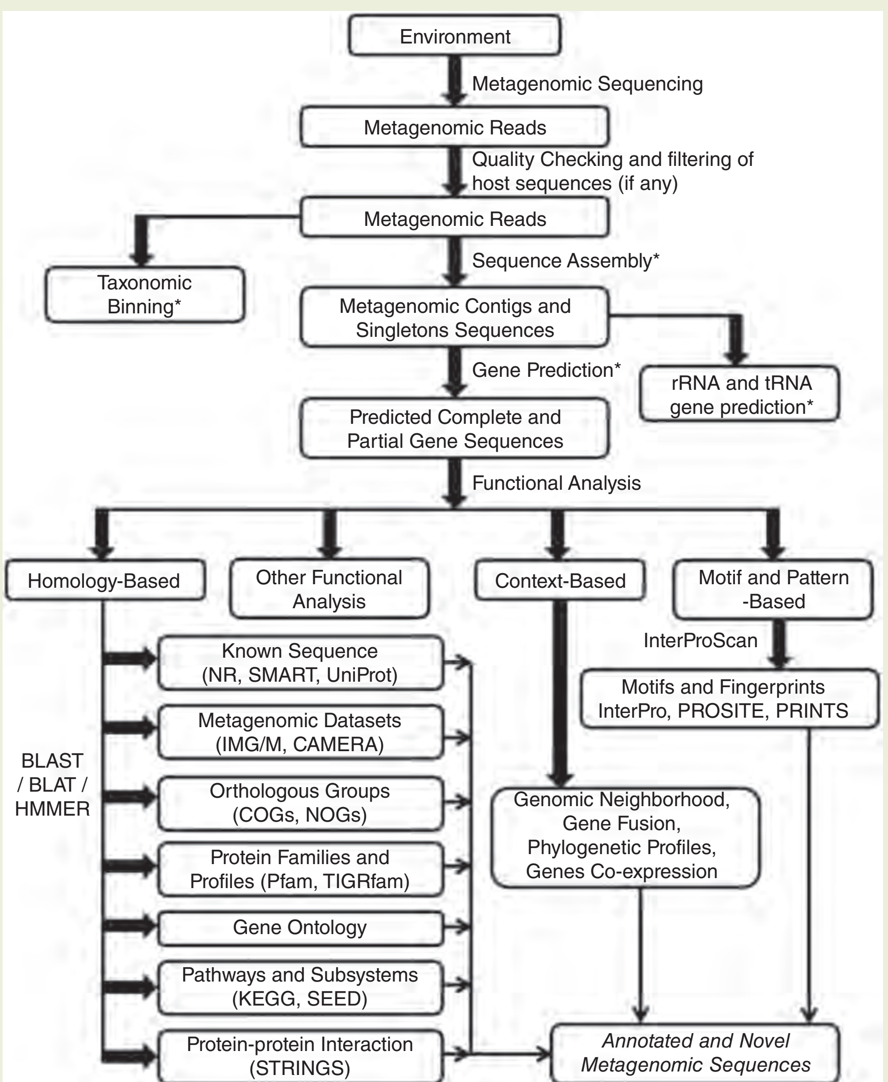


Figure 1 Flow chart for the analysis of a metagenome from sequencing to functional annotation. Only the basic flow of data is shown up to the gene prediction step. For the context-based annotation approach, only the gene neighborhood method has been implemented thus far on metagenomic data sets, although, in principal, other approaches that have been used for whole genome analysis can also be implemented and tested. See text for further details. *Abbreviations*: NR = non-redundant; SMART = Simple Modular Architecture Research Tool; IMG/M = Integrated Microbial Genomes/Metagenome; CAMERA = Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis; COG = Clusters of Orthologous Groups (of proteins); NOG = Nonsupervised Orthologous Groups; KEGG = Kyoto Encyclopedia of Genes and Genomes; SEED = Fellowship for the Interpretations of Genomes (FIG)-created single resources integrating Genome databases; STRING = Search Tool for the Retrieval of Interacting Genes; PROSITE = database of protein domains, families and functional sites; PRINTS = compendium of protein fingerprints; InterPro = Integration of protein predictive models based on signature pattern domains; Pfam = database at NCBI of conserved protein families; TIGRfam = The Institute for Genome Research program that relies on its own protein database to annotate new sequences. (Reproduced with permission from Oxford Journals; from Prakash, T, and T.D. Taylor. 2012. Functional assignment of metagenomic data: challenges and applications. *Briefings in Bioinformatics* **13**: 711–727.)

Box 6.6 *Continued*

packages appear in *italics*): (i) quality control and filtering of noise (e.g., *PyroNoise*), (ii) sorting ("binning") of reads (e.g., *TETRA, MEGAN*), (iii) sequence assembly (e.g., *Phrap, Newbler, JAZZ, Velvet*); (iv) taxonomic categorization (e.g., *EMIRGE, MetaBin, NBC, TACOA, Mothur, QIIME*), and (v) gene prediction (e.g., *BLAST, GLIMMER, ORF-Finder, GeneMark*).

The need to integrate the many gene assembly and analysis steps shown in Figure 1 has been addressed by publically available pipelines that use multiple sequential software packages and databases to interpret metagenomic data. Table 1 (from Prakash and Taylor, 2012) provides an overview of eight well-recognized web-based data-processing platforms (and their internet URL addresses) that are essential tools for making sense of metagenomic data. For each of the eight pipelines shown, Table 1 provides an overview of featured computational approaches. Among the key entries within Table 1 are several critical databases with associated search and analysis capabilities, which are pivotal for bioinformatic processing of metagenomic data: the massive nonredundant (NR) GenBank gene compilation at the National Center for Biotechnology Information (NIH, USA), Simple Modular Architecture Research Tool at the European Molecular Biology Laboratory (Germany), the Universal Protein Resource developed by both the European Bioinformatics Institute and the Swiss Institute for Bioinformatics, the Kyoto Encyclopedia of Genes and Genomes at Kyoto University in Japan, and the Search Tool for Retrieval of Interacting Genes/Proteins.

- **Question:** When millions of sequencing reads are processed, what does the information look like?
- **Answer:** Trends are often discerned by representing the data as "heat maps".

Vast numbers of sequencing reads are sorted, quantitatively analyzed, and interpreted according to investigation-specific experimental designs. Figure 2 presents a heat map depicting the response of gut microbiota to diet-influenced inflammation in mouse tissue. The experimental design quantitatively monitored mRNA-transcripts (expression) of 10 genes (from *F4/80* (top) to AngPt14 (bottom) of the *y* axis in Figure 2) in mice and correlated the transcription levels of these inflammation-related genes with the relative abundances of 18 types (taxa) of microorganisms found in the mouse gut (from *Actinobacteria*, *x* axis (far left), to *Verrucomicrobia* (far right) in Figure 2). Patterns in the ranges of colors in Figure 2 (blue is "cold" and red is "hot") reveal 12 clear strong positive correlations (red boxes with "+" or "++") in which high abundances of particular bacterial taxa are associated with high expression of particular genes in mouse tissue. Based on these patterns, biochemical and/or physiological mechanisms can be hypothesized and tested – linking particular members of the resident gut microbiota and the dietary and/or inflammatory status of the host.

In the future, metagenomic data sets describing microbial communities are likely to become increasingly large and complex and these data sets may be interactive – allowing the viewer to interrogate the data from multiple viewpoints – to discover new relationships. Figure 3 provides a glimpse of how bioinformatics may transform some areas of environmental microbiology in the future. Shown is a "screen shot of a three-dimensional heat map" of changes in the microbial community composition in the gut of human infants over time. The names of individual microbiological taxa are illegible and they disappear into the back, right-hand side of the figure: abundances of individual taxa are charted on the vertical axis. Along the front of Figure 3, the experimental design is revealed as "Infants 1,2,3,4,5, and 6 having their gut microbiomes sampled weekly for 8,5,5,3,3, and 3 weeks, respectively. Dynamic trends among microbial populations are apparent by inspecting the colors and heights of the bars" (Foster et al., 2012).

Table 1

Representative list of commonly used publicly available pipelines for the functional annotation of metagenomic data sets. (Reproduced with permission from Oxford Journals; modified from Prakash, T, and T.D. Taylor. 2012. Functional assignment of metagenomic data: challenges and applications. *Briefings in Bioinformatics* **13**:711–727)

Pipeline/ tools	IMG/M	METAREP	CAMERA	MG-RAST	Smash community	MEGAN4	CoMet	WebMGA
Functional analysis								
• Homology-based known sequence	NCBI (NR), SMART, Uni Prot	NCBI (NR) Uni Prot	—	NCBI (NR) SMART, UniProt. IMG/M	SMART, UniProt	NCBI (NR)	—	NCBI (NR)
• Metagenomic data sets	IMG/M	—	—	IMG/M	—	—	—	—
• Orthologous groups	COGs	—	COGs	COGs, eggNOGs	COGs, eggNOG	—	—	COGs
• Protein families	Pfam, TIGRfam	Pfam, TIGRfam	Pfam, TIGRfam	FIGfams	Pfam	—	Pfam	Pfam, TIGRfam
• Ontology	GO	GO	GO	GO	—	—	GO	GO
• Enzymes, pathways, and subsystems	KEGG, SEED	PRIAM	KEGG, SEED	KEGG, SEED	KEGG	KEGG, SEED	—	KEGG
• Protein interactions	—	—	—	STRING	STRING	—	—	—
• Motif- and pattern-based database	InterPro	—	—	—	—	—	—	—

Table 1 *Continued*

Pipeline/ tools	IMG/M	METAREP	CAMERA	MG-RAST	Smash community	MEGAN4	CoMet	WebMGA
Functional analysis								
• Context-based approach	Gene neighborhood	—	—	—	Gene neighborhood	—	—	—
• Other functional analysis types of prediction	CRISPRs enzymes transporter classes	Enzymes, transmembrane helices, lipoprotein motifs	—	—	Protein networks	—	—	—
• URL	http://img.jgi.doe.gov/cgi-bin/m/main.cgi	http://www.jcvi.org/metatrep/	http://camera.calit2.net/index.shtm	http://metagenomics/anl.gov/	http://www/bork.embl.de/software/smash/	http://ab.inf.uni-tuebingen.de/software/megan/	http://comet.gobics.de/	http://weizhong-lab.ucsd.edu/metagenomics.ucsd.edu/metagen-analysis/server/hmr

IMG/M = Integrated Microbial Genomics/Metagenomics, USDOE; METAREP = Metagenomic Reports, JC Venter Institute; CAMERA = Community Cyberinfrastructure for Advanced Microbial Research and Analysis; MGRAST = MetaGenomic Rapid Annotations using Subsystems Technology; MEGAN = MetaGenome Analyzer; CoMet = comparative metagenomics; wbMGA = Web MetaGenomics Analysis; NCBI = National Center for Biotechnology Information (National Institutes of Health, USA); NR = nonredundant; SMART = Simple Modular Architecture Research Tool (European Molecular Biology Laboratory, Germany); UniProt = Universal Protein Resource (European Bioinformatics Institute and Swiss Institute for Bioinformatics); COG = Clusters of Orthologous Groups (for proteins); eggNOGs = evolutionary genealogy of genes, Non-supervised Orthologous Groups; Pfam = protein families database at NCBI; TIGR = The Institute for Genome Research; GO = gene ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes; SEED = integrates, many publically available genome databases into single resource; PRIAM = Profiles for the Automatic Identification of enzyme-specific Metabolism; STRING = Search Tool for the Retrieval of Interacting Genes/Proteins; CRISPRs = Clustered Regulatory Interspaced Short Palindromic Repeats.

Box 6.6 *Continued*

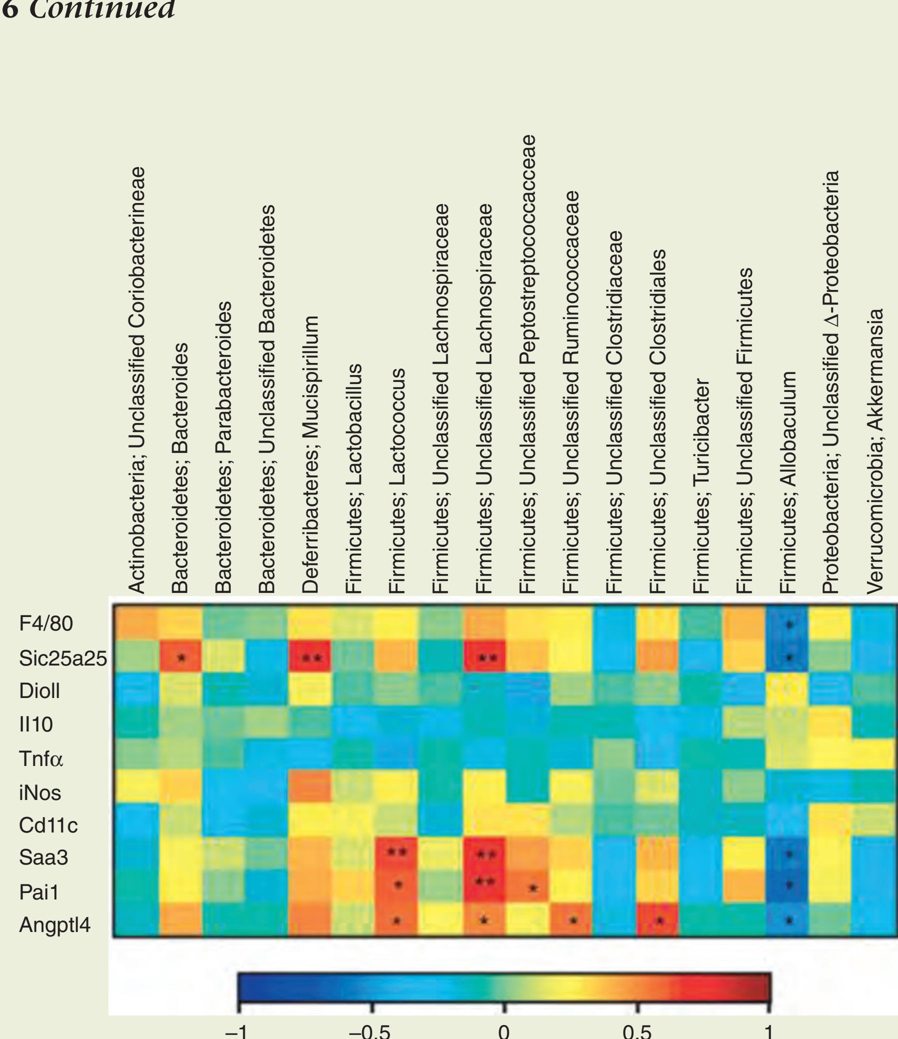


Figure 2 Heat map describing the correlation of the abundances of 18 different bacteria in the mouse gut and transcription levels of inflammation-related mouse genes in inguinal adipose tissue. The colors range from blue (negative correlation; –1) to red (positive correction; 1). Significant correlations are noted by $^{*}P < 0.05$ and $^{**}P < 0.01$). (Reproduced with permission from John Wiley and Sons; from Ravussin, Y, O. Koren, A. Spor, C. LeDuc, R. Gutman, J. Stombaugh, R. Knight, R.E. Ley, and R.L. Leibel. 2012. Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obesity* **20**:738–747.)

Box 6.6 *Continued*

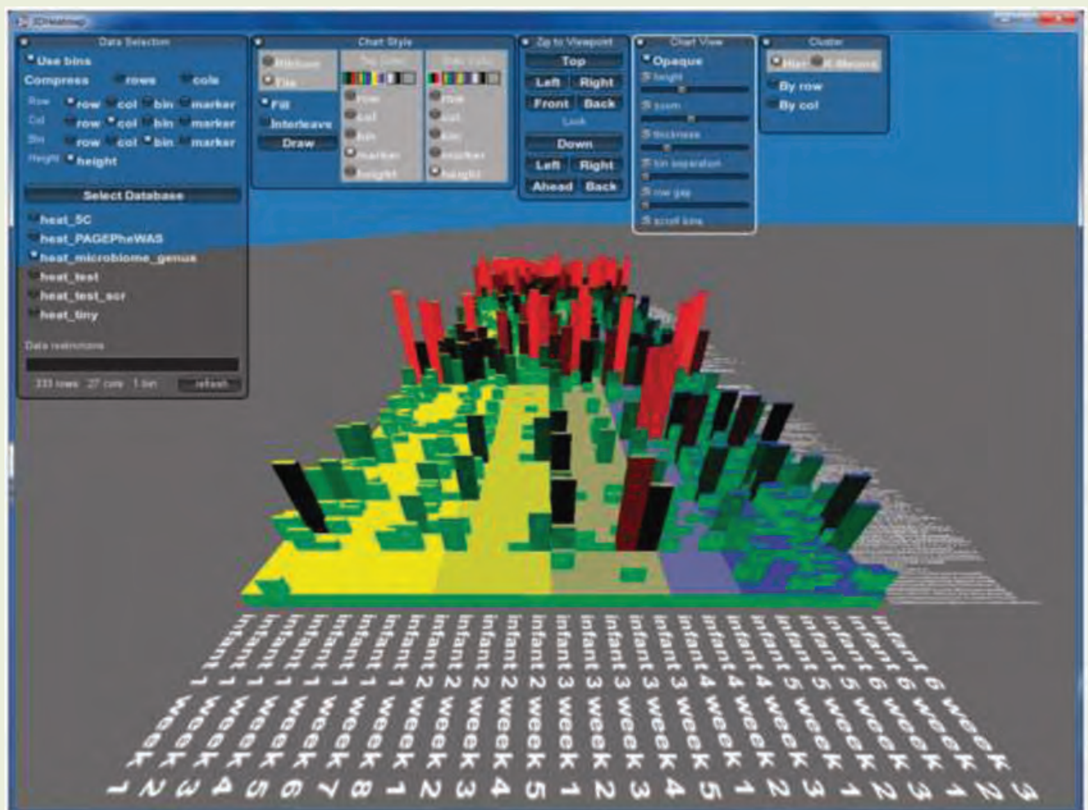


Figure 3 Screenshot of the three-dimensional heat map application showing menus for data selection, chart style, viewpoint, chart view and cluster analysis. Each menu can be minimized or hidden. Illustrated are human microbiome data. Each row is a microbe with the name shown on the *y* axis. Each column is a different subject and time point. The *z* axis represents the relative abundance of the microbes. The three-dimensional heat map makes it possible to add additional layers of information in the fourth and fifth dimensions, using colors. (Reproduced with permission from Oxford Journals; from Foster, J.R., J. Bunge, J.A. Gilbert, and J.H. Moore. 2012. Measuring the microbiome: perspectives on advances in DNA-based techniques for exploring microbial life. *Briefings in Bioinformatics* **13**:420–429.)

DNA Sequencing technology: from classic Sanger sequencing to next-generation sequencing

It is important to understand relationships between the technologies that allow scientific information to be created and the developments spurred by that information. Table 6.8 provides an overview of DNA sequencing technologies; short descriptions of each technology (and others) appear in Box 6.5.

Key characteristics of all sequencing technologies include accuracy (Table 6.8, rows 6 and 7) and read length (Table 6.8, row 9). Regarding accuracy, the classic Sanger sequencing technology exceeds that of next-generation technologies (Table 6.8, Column 9). Reads are the fundamental units of information created by each technology: each read is a single sequence of nucleotides (A, T, G, C, ...) created from DNA or RNA originating from an organism in an environmental sample. In matching unknown sequences (reads) to those in existing databases, the robustness of a match is proportional to the read length. For the assembly of overlapping reads into longer contigs and scaffolds ("sewing" many short reads together into genomic assemblies), the longer the read length, the easier it is for the genomic puzzle pieces to fit together. Again, many next-generation technologies fall short of the gold standard (~800 bp) for read length set by classic Sanger sequencing (the major exceptions are PacBio RS, 454 GS FLX; Illumina is constantly extending its read-length capabilities; see Table 6.8). Another characteristic of each technology is the "number of sequencing reads per analytical run". To calculate this, simply divide the total sequencing yield per run (Table 6.8, row 4) by the average read length (Table 6.8, row 9). Using this arithmetic, 454 GS FLX produces $\sim 10^6$ reads per run (0.7 Gb/run ÷ 700 bp/read), while Illumina HiSeq 2500 produces $\sim 3 \times 10^9$ reads per run (1000 Gb/run ÷ 300 bp/read).

Though the application of next-generation sequencing power to environmental microbiology has already produced a new world of "deep sequencing data", the reader should be aware of at least two caveats already mentioned above: accuracy and read length. Compared to Sanger sequencing, next-generation sequencing technologies feature short read lengths and/or low accuracy. *These characteristics make next-generation-derived data sets inherently "blurry"* (e.g., Temperton and Giovannoni, 2012). Though broad trends across millions of sequences can be incredibly insightful (see below), no single read is as robust or rigorous or valid as a comparable read (often confirmed via redundant sequencing of both "plus" and "minus" DNA strands) obtained from Sanger methodology (see Box 6.6).

An obvious way to validate the application of next-generation sequencing approaches to complex naturally occurring communities is to prepare

Table 6.8
Technical specification of available next-generation sequencing platforms. (From Quail et al., 2012; Liu et al., 2012; Loman et al., 2012; Mardis, 2013)

Platform	454 GS FLX	Illumina HiSeq 2500	Illumina GAIIx	Illumina Mi Seq	PacBio RS	Ion Torrent PGM	SOLiD	Sanger 3730 × 1
Instrument cost*	$500K	$654K	$256K	$128K	$695K	$80K[†]	$350-495K	$95K
Sequencing mechanism	Sequencing by synthesis – pyrophos-phate stimulation of light	Sequencing by synthesis – nucleotide incorporation stimulation of light	Sequencing by synthesis – nucleotide incorporation stimulation of light	Sequencing by synthesis – nucleotide incorporation stimulation of light	Single molecule monitoring of individual bases added to growing strand	Sequencing by synthesis – base incorporation leads to proton release	Ligation and two-base coding yields fluorescent signals indication of template	Dideoy chain termination
Sequence yield per run	0.7 Gb	1000 Gb	30 Gb	12 Gb	100 Mb	20–50 Mb on 314 chip, 100–200 Mb on 316 chip, 1 Gb on 318 chip	120–159 Gb	1.9–84 Kb
Sequencing cost per 10^9 bases*	$10K	$40–$70	$150–$200	$502	$2000	$1000 (318 chip)	$130	$2·10⁶
Run time	10–24 h	3–10 days	10 days	27 h[‡]	2 h	2 h	7 days for SE 15 days for PE	20 min to 3 hrs
Reported accuracy	99.9%	Mostly >Q30, 98%	Mostly Q30	Mostly >Q30	<Q10	Mostly Q20	99.94%	99.999%

Observed raw error rate		0.26%	0.76%	0.80%	12.86%	1.17%		0.001
Read length	700 bases	Up to 600 bases	Up to 600 bases	Up to 600 bases	Average 1500–3000 bases; up to 10 Kb§ (C1 chemistry)	120–200 bases	50 + 35 or 50 + 50 bases	700–900 bases
Paired reads	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Insert size		Up to 700 bases	Up to 700 bases	Up to 700 bases	Up to 10 kb	Up to 250 bases		
Typical DNA requirements		50–1000 ng	50–1000 ng	50–1000 ng	~ 1 µg	100–1000 mg		
Cost per run	$7000	$6000	$3000	$1200	$400	$1200	$15,000	$4 for 800 bp reaction

*All cost calculations are based on list price quotations obtained from the manufacturer and assume expected sequence yield stated.

†System price including PGM, server, One Touch, and One Touch ES.

‡Includes two hours of cluster generation.

§Mean mapped read length includes adapter and reverse strand sequences. Subread lengths, i.e., the individual stretches of sequence originating from the sequenced fragment, are significantly shorter. PE = paired end read; SE = single-end read; Gb = 10^9 base pairs.

a synthetic mixture of known microbial populations (for example 10 pure-culture bacteria added in equal numbers to a sterile buffer medium) and then compare the output of the next-generation technology of interest to the empirically defined, known synthetic community composition. When Quince et al. (2011) implemented such a study, the results were rather shocking; after preparing a mixture of 90 cloned bacterial 16S rRNA sequences, amplifying a portion of this gene by PCR, and then analyzing the pool of DNA using 454 pyrosequencing technology, ~10^4 "species" (actually operational taxonomic units (OTUs); see Section 5.3) were found. This stunning (100-fold) overestimation of diversity is now accepted as "noisy data" or "pyronoise" (because the noise was created by the 454 pyrosequencing technology itself). Please recall, however, that the criteria for making judgments of distinctive OTUs (or "species") are somewhat subjective and variable (see Section 5.3). The tally of ~ 10^4 OTUs found by Quince et al. (2011) in the mixture known to have only 90 OTUs could be calibrated downward by altering the standard 3% cut-off criterion routinely used to categorize 16S rRNA sequences as differing from one another at the species level. When the cut-off was raised to 8% sequence difference, then the output from pyrosequencing far better matched the expected outcome of 90 bacterial 16S rRNA sequences. This type of bioinformatic "de-noising" procedure is now necessary and routine in interpreting the output of next-generation-based technologies when used to characterize complex microbial communities (Quince et al., 2011; Kunin et al., 2010; Braun et al., 2010; Gilles et al., 2011; Degnan and Ochman, 2012; Gloor et al., 2010).

It is important to note that the noise issue is significantly diminished when next-generation technologies are applied to genomic sequencing of pure cultures of microorganisms; this is because the massive data sets and thorough, redundant, coverage of single genomes compensate for noise. Full genomic assembly is facilitated because all the pieces of a single coherent genome are initially present in the sequencing reaction. By contrast with complex microbial communities (~10^3 to 10^6 genomes, or more, per sample), redundancy and overlap in sequencing coverage for a given member of the microbial community cannot be high. Under such circumstances, assembly of genomes is a rare occurrence: the vast majority of sequencing reads are "orphans" without a tie to a contig, or to a scaffold, or to a genome. Thus, in highly complex communities, valid reads (the "signal" being sought) and artifacts of the sequencing technology (noise) often cannot be easily distinguished. Indeed, early investigations of complex naturally occurring communities claimed to discover thousands to millions of 16S rRNA sequences that were novel – leading to claims of discovering the "rare biosphere" (Sogin et al., 2006; Huse et al., 2010; Bender and Knight, 2009; Zhou et al., 2011b). While it is likely that large proportions of naturally occurring microbial communities are composed of numerous

novel populations in low abundance, noisy sequencing technologies are the wrong tools for finding the evidence (the signals) for rare microorganisms.

Barcode-based sequencing of small-subunit rRNA genes

- **How do we take a census of "who is there" in complex, naturally occurring microbial communities?**

Answering this question is fundamental to environmental microbiology. For this reason, approaches addressing this question emphasizing small subunit rRNA genes have appeared in Sections 5.2, 5.3, 5.4, 5.10, and 6.7. Certainly next-generation sequencing technologies can make a contribution!

Figure 6.7 provided an overview of nucleic-acid based characterization of the composition of microbial communities. While cloning-based approaches were featured in Section 6.7, the right-hand portion of Figure. 6.7 alluded to PCR-linked next-generation sequencing. Given the potential for next-generation DNA sequence technology to produce enormous amounts of data (Table 6.8), it was inevitable that this sequencing power be applied to microbial communities via analysis of small-subunit rRNA genes. Here we discuss this approach, which begins with producing libraries of PCR-amplified small-subunit rRNA genes and culminates the analysis with next-generation sequencing. Note that the narrowed scope of barcode-based sequencing of small-subunit rRNA genes departs from the broad scope of true metagenomic methodologies (see Section 6.9).

- **What does "barcode-based" sequencing mean?**

"Barcoding" (formally known as "Golay barcoding") is a strategy that incorporates a string of 6–12 nucleotides (A, T, G, C) in distinctive order into the 3′ end of PCR primers targeting a given gene (in this case, small subunit rRNA). The PCR-amplified mixture of small-subunit rRNA genes from a DNA extract of a microbial community *represents* the composition of community members and each sequence within a given amplified pool is tagged with a common barcode fixed into all members of the pool by each set of PCR primers. The potential for distortion of community composition via "PCR bias" (discussed in Section 6.7) is inherent in barcode-based sequencing of small-subunit rRNA genes. Implicit in an investigator's choice to use this technique is a judgment that potential distortion of the data set is outweighed by the benefit of thorough sampling of community composition. In this regard, the Human Microbiome Project (HMP) (2012) compared phylum-level community compositions of microbiomes

occupying seven human body sites using both barcode-based 16S rRNA gene sequencing and whole-genome shotgun sequencing: broad trends in community composition were shared between both data sets, but significant discrepancies (distortions) were also evident in the barcoded data.

All the next-generation sequencing procedures described above and in Table 6.8 are capable of creating millions of sequencing reads and all are expensive for a single sequencing reaction run – especially compared to the ~$4-per run cost of Sanger sequencing. Barcoding allows multiple pools of DNA amplicons (each prepared from a separate PCR reaction using a distinctive barcode or "tag") to be sequenced in a single sequencing reaction run (Hamady et al., 2008). The data output from such a run is bioinformatically sorted according to the barcode tags prior to compositional analysis (matching of new sequences to catalogued database sequences). In the original study by Hamady et al. (2008), as many as 288 pools of PCR amplicons were shown to be processed in a single sequencing run; thus, if the 454 pyrosequencing platforms were being used (~10^6 reads/run), ~3500 reads/sample (~10^6 reads/run ÷ 288 samples/run) may be obtained for each of the 288 samples processed in a single run. PCR-based barcoding systems have also been developed for the Illumina sequencing platform (e.g., Bartram et al., 2011; Gloor et al., 2010) – allowing multiple batches of PCR-amplified gene pools to be sequenced *en masse* and to later be bioinformatically sorted. Whenever a sequencing technology yielding short read lengths is used, the interrogated region of the small subunit rRNA gene (PCR target) needs to be appropriately sized. Section 5.5 explained the stem-loop secondary structure of 16S- and 18S-rRNA molecules; portions of these molecules known as "V6", "V5", and "V3" are the typical PCR target for Illumina barcode-based census taking (Zhou et al., 2011a; Caporaso et al.).

Table 6.9 provides several detailed examples of how barcoding has been combined with next-generation sequencing to characterize the composition of naturally occurring microbial communities. The first entry in Table 6.9 was one of the earliest attempts to use barcoded Illumina sequencing (~150 bp reads) to assess microbial diversity in soil; samples from the Arctic tundra were examined. Phylum (e.g., *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*), class (e.g., *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*), and order (e.g., *Acidobacteriales*, *Burkholderiales*, *Chromatiales*) level relative abundances of *Bacteria* were reported. Much effort was devoted by Bartram et al. (2011) to quality control in the data, especially "artifactual sequences" leading to information "only useful at a coarse (taxonomic) level".

Entry number two in Table 6.9 shows results of a thorough exploration of microbial life dwelling at the root–soil interface. Bulgarelli et al. (2012) used 454 pyrosequencing of barcoded 16S rRNA genes (~400-bp reads) to catalog patterns in the occurrence of soil *Bacteria* associated with roots of *Arabidopsis* plants. Rarefaction analysis (see Section 5.3) suggested that sampling of OTUs (≥97% similarity) was reasonably thorough and that bulk soil (in the absence of a plant) featured higher diversity (~2000 distinct sequences) than soil in the root zone (~1000 distinct sequences).

Table 6.9

Examples of next-generation barcode-based small subunit rRNA investigations: their strategies, goals, and outcomes

Habitat sampled	Goal	Main sequencing technology	Accessory community composition assay	Number of samples	Total DNA sequenced	Outcome	References
1. Arctic tundra soil	Validate application of bar code Illumina sequencing by completing assay on synthetic community of 6 bacteria and on uncharacterized Arctic soil	Illumina GAII x (paired end); V3 region of 16S rRNA gene	Sanger sequencing of 16S rRNA clone libraries	4 (2 samples, each in duplicate)	~1.1 Gb (13.8×10^6) reads trimmed to 7.5×10^6; average read length, 150 bp	Phylum-, class-, and order-level census of soil community achieved. Artifactual sequences limit community characterization to coarse taxonomic levels	Bartram et al., 2011
2. Root–soil interface (rhizosphere) of field-grown *Arabidopsis thaliana* plants	Explore patterns in colonization of plants roots by soil microorganisms. Do plants deliver cues to root-associated bacteria?	454 pyrosequencing (titanium); V5, V6, V7 region of 16S rRNA gene	Scanning electron microscopy of root surface, as well as 16S rRNA-based fluorescent in situ hybridization to visualize key bacteria on root surface	122 (2 agricultural sites, 3 locations in each soil (bulk soil, rhizosphere, and root tissue), with replication)	~0.23 Gb; 400 bp read lengths trimmed to 315 bp	Roots of *Arabidopsis* plants are preferentially colonized by soil-type-specific *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Cues for colonization are both plant-specific and driven by generic plant tissues undergoing decay	Bulgarelli et al., 2012

Table 6.9 *Continued*

Habitat sampled	Goal	Main sequencing technology	Accessory community composition assay	Number of samples	Total DNA sequenced	Outcome	References
3. Human gut microbiome viewed against age and global geography	Characterize bacterial species in fecal samples from 531 healthy children and adults in Amazonas Venezuela, Malawi, and the US	Illumina HiSeq 2000; V4 region	Metagenome of fecal samples from 110 people	531 people	5.9 Gb (total of all sequencing); 1 billion reads from the V4 region; 123 bp/read	Phylogenetic composition of gut bacterial communities evolve toward the adult configuration from birth to age 3; interpersonal variation in gut community composition is higher among children than adults; gut microbiota vary substantially from country to country	Yatsunenko et al., 2012

By comparing patterns among the taxa of *Bacteria* between the three zones (bulk soil, rhizoplane, and microorganisms tightly associated with root tissue), across different soil types, Bulgarelli et al. (2012) concluded that the plant provides major cues that specifically enrich for certain classes of *Bacteria* on their roots.

The third entry in Table 6.9 displays results of a study using Illumina-based barcoded 16S rRNA sequencing (123 bp reads) to survey human gut *Bacteria* sampled from healthy individuals across many ages (from children to adults) and across three countries (Amazonas Venezuela, rural Malawi, and urban US). The goal was to explore commonalities and contrasts in human gut microbial communities. Results emphasized the importance of key factors (especially microbial exposure history, kinship, diet, and age) in shaping gut microbial ecology.

Next-generation metagenomic investigations

As discussed in Section 6.9, *metagenomics* expands the view of community composition from interest in taxonomically insightful small subunit rRNA genes to the entire complement of structural, regulatory, and all other types of genes (see Section 3.2) that contribute to whole-cell function. Figure 6.7 (right-hand side) shows one path of data generation that eliminates all cloning and PCR steps; instead, environmental DNA is directly processed via next-generation sequencing technology. Summarized in Table 6.10 are five examples of landmark next-generation-based metagenomic investigations. Note the vast size of data sets (millions to billions of reads, comprising 10^9 (giga) to 10^{12} (tera) bases of sequences) generated in these studies (column 6, Table 6.10).

- **Question:** How can the immense data sets described in Table 6.10 be interpreted and understood?
- **Answer:** Bioinformatics! (and graphical data interpretations using the "heat maps" described in Box 6.6).

The five entries in Table 6.10 were selected because they illustrate a range of questions that can be asked in environmental microbiology using representative next-generation sequencing technologies. Delmont et al. (2012; entry 1) aimed to characterize soil microbial and genetic diversity by applying 454 pyrosequencing to DNA extracts of 9 main samples surveying 3 depths at the Rothamstead Field Station (UK) at 3 times spanning 1.5 years. The MG-RAST pipeline (see Box 6.6) was used to map >12 million reads into 835 metabolic subsystems; for community composition, recovered small-subunit rRNA genes were also analyzed. Findings from Delmont et al. (2012) emphasized enrichment of the soil genome in functional genes involved with S, P, and K metabolism, as well as membrane transport, stress response, and cell–cell signaling. These investigators

Table 6.10

Examples of next-generation sequencing-based Metagenomic investigations: their strategies, goals, and outcomes

Habitat sampled	Goal	Main sequencing technology	Accessory community composition assay	Number of samples	Total DNA sequenced	Outcome	References
1. Grassland soil (Rothamstead, UK)	Characterize soil microbial and genetic diversity	454 Pyrosequencing FLX Titanium	None	9 (3 depths, 3 time points over 1.5 years) plus 4 auxiliary samples	4.88 Gb; 12,575,129 reads; average read length, 386 bp	Assembly of reads into contigs was attempted; most were 500 bp or shorter, though 10 Kbp contigs were obtained; 34.5% of the reads could be annotated (using MG-RAST); these were binned among 835 functional metabolic systems. <1% of genes mapped to sequenced genomes. 88.6% were bacterial, 0.91% were eukaryotic, and 1.4% were archaeal. *Alpha-*, *Beta-*, *Gamma-Proteobacteria* and *Actinobacteria*; and three genera (*Bradyrhizobium*, *Rhodopseudomonas*, and *Nitrobacter*) were dominant	Delmont et al., 2012
2. Surface marine water (Puget sound, USA)	Characterize marine microbial community	SOLiD	Cloning of 264 16S rRNA genes	2	58.5 Gb; 1171 million reads; average read length 47–48 bp	Extensive sequencing coverage allowed assembly of 14 candidate genomes. The genome of an uncultured member of marine Euryarcheota was assembled, revealing reliance on photoheterotrophy and metabolism of both proteins and lipids. Early proteorhodopsin evolution was revealed	Iverson et al., 2012

3. Cow rumen (microbial community bound to switchgrass plant material)	Discover new genes and enzymes and microbes	Both 454 pyrosequencing and Illumina	Barcoded 454 pyrosequencing of 16S rRNA genes; validate biochemical function of 90 putative new carbohydrate-active genes using expression assays	4	268 Gb; 1.5 billion reads; average read length 72-221 bp	Focus on 27,755 genes encoding carbohydrate-active enzymes. Most were novel. 90 of these were expressed to verify function. After tetranucleotide-based binning of reads, 15 draft genomes were assembled. Validity of one draft genome was verified using whole-genome amplification and sequencing	Hess et al., 2011
4. Groundwater anaerobic microbial community (Rifle, Colorado USA)	Explore novel genetic and physiological diversity	Illumina GAIIx	Reconstuction of 16S rRNA from Illumina reads; also 16S rRNA clone libraries and meta-proteomics	3	19.1 Gbp; 232 million reads; average read length 85 bp	Using a tetranucleotide-based binning scheme, emergent self-organizing maps (ESOMs) allowed assembly of 87 draft genomes, 49 described here. Two genomes were from uncharacterized microbial phyla; one was from a new evolutionary lineage. All were nonrespiring anaerobes predicted to ferment, and/or to carry out hydrogen and sulfur metabolism	Wrighton et al., 2012
5. Human bodies (18 body sites: gut, oral cavity, airways, skin, vagina) of 242 healthy adults	Characterize the microbial residents of the human body	Illumina GAIIx and 454 Pyrosequencing	Barcoded 454 pyrotag sequencing of amplified 16S rRNA genes (5,298 samples)	681 (Illumina); 12 (454); 560 processed for both 16S rRNA and metagenome	8863 Gb; trimmed to 3500 Gb; 454 read length > 300 bp; Illumina read length 101 bp	49% of the reads were from human genes. After removal of these, 57.6% were associated with known microbial genomes; 99.7% of these were *Bacteria*. Major phyla (and key respective habitats) include: *Firmicutes* (all; mouth, nose, skin, gut, vagina), *Actinobacteria* (skin, nose), *Bacteroidetes* (gut), *Proteobacteria* (mouth), and (skin, nose, mouth). Comprehensive compilation of reference data	Human Micro-biome Project (HMP), 2012

estimated that ~450–454 pyrosequencing runs would be required to create contigs among all of the DNA fragments they found. In other words, the soil community they found was extremely complex, genetically – thorough sampling would require a sequencing depth several hundredfold greater than was applied in this study.

Iverson et al. (2012; second entry in Table 6.10) used deep metagenomic sequencing to examine the surface seawater microbial community in Puget Sound (Washington State, USA) in October 2008 and May 2009. Extracted DNA was analyzed using SOLiD technology (~1 billion reads, 47–48 bp in length). Despite the short read length characteristic of SOLiD technology, contigs and scaffolds were assembled into 15 candidate genomes, each representing 4–10% of the sampled community. A closed genome of a novel member of the Group II *Euryarchaeaota* revealed photoheterotrophy to be its physiological way of life: this member of the marine *Archaea* is motile, likely to utilize proteins and lipids as C sources, and supplements its energy budget using proteorhodopsin (see Box 6.4).

In an effort to expand knowledge of genes that encode cellulose-degrading enzymes, Hess et al. (2011; entry 3, Table 6.10) completed an extremely thorough, multidisciplinary examination of the microbial community degrading switch grass in the rumen of a cow. Both Illumina and 454 pyrosequencing technologies generated the DNA sequences (268 Gb; 1.5 billion reads). New diverse carbohydrate-active genes were discovered (only 12% of 27,755 genes matched known sequences at >75% identity). Fifteen draft genomes were assembled. Validation of bioinformatics-based interpretation of sequencing data relied upon both biochemical assays of genes (cloned and expressed in laboratory hosts) and single-cell whole-genome amplification and sequencing. Wrighton et al. (2012; entry 4 of Table 6.10) used deep (Illumina) sequencing to characterize the microbial community residing in anoxic aquifer sediments contaminated with uranium. A novel system of bioinformatics processing (tetranucleotide-based binning in combination with "emergent self-organizing maps") allowed the assembly of 49 draft genomes of uncultivated, previously unrecognized bacteria whose metabolic pathways indicate physiologies reliant upon fermentation and the metabolism of hydrogen and sulfur. Advances in bioinformatic tools capable of assembling genomes of uncultivated microorganism from community metagenomic data are ongoing. For example, Albertsen et al. (2013) generated 31 population genome bins from metagenomic sequencing of an activated-sludge bioreactor; the result was 12 complete or near-complete bacterial chromosomes (validated as carrying single copies of known single-copy genes and carrying the full complement of 107 proteins conserved in 95% of all *Bacteria*).

The final entry in Table 6.10 (Human Microbiome Project, 2012) used both 454 pyrosequencing and Illumina technologies to assess the gene content of microbial communities dwelling in or on humans. This comparative study compiled data from many investigators surveying the

metagenomes of 18 human body sites (from gut, to oral cavity, to vagina, to skin) across 242 healthy human adults. The goal here was to establish baseline information about the composition of the microbial communities that inhabit the human body (that constitute the "human microbiome") so that mechanistic relationships between our microbiome and human health and disease can eventually be discovered, managed, and exploited by the medical profession.

Note that barcode technology (that allows multiple pools of 16S rRNA amplicons to be sequenced in a single next-generation run; see above) has also been applied to metagenomic analyses. In this case, distinctive oligonucleotide tags are uniformly ligated to all DNA fragments in a given DNA extract bound for metagenomic sequencing. Multiple DNA pools so labeled can be combined and then the sequenced reads can be bioinformatically separated from one another prior to detailed analyses. This barcode labeling system is not PCR-based; instead it relies upon attaching the barcode tags to blunt-end-repaired DNA via ligation and strand displacement.

Next-generation metatranscriptomics investigations

Knowing the fundamental genetic architecture (DNA blueprint) of a microorganism (an entire genome) or microbial community (metagenome) establishes "the players and their potential". However, it is certain that a large proportion of the players and/or their respective genomic pools in every sampled naturally occurring microbial community are *dormant* (entirely or largely shut down; see Section 3.5). It follows, then, that one must be cautious when interpreting information describing the full metagenome. To discover actual, ongoing, ecologically relevant metabolic activity, we need a refined assay that accesses the pool of genes actively transcribed into mRNA. This provides a window into site-specific, real-time metabolic activity manifest as both cellular house-keeping (e.g., DNA replication, membrane biosynthesis, ATP synthesis) and biogeochemical (e.g., nitrogen fixation, sulfate reduction, iron reduction) processes. Thus, the key motivation for metatranscriptomic assays is to find out "what the microbial communities are doing", to assess "community phenotype" (Moran et al., 2013). In their recent appraisal of metatranscriptomics, Moran et al. (2013) have stated that "instantaneous inventories of mRNA polls are … highly informative about ongoing ecologically relevant processes". This largely reflects short (~1–8 minute) intracellular half lives that closely link mRNA expression to the physiological or environmental cues that trigger them. These same authors also offer cautionary advice about potential erroneous conclusions that may follow from mRNA data: "The abundance of mRNAs from functional genes is *not* a reliable rate proxy for these functions in naturally fluctuating environments." This lack of reliability is caused by imperfect coupling of mRNA transcription, translation of the corresponding protein, assembly of functioning metabolic pathways, and integrated cell physiology. Despite the need to

carefully interpret environmental metatranscriptomics, the allure of assessing community gene expression in real time is extremely strong. Table 6.11 provides four key examples of transcriptome-based inquiry. All have a shared methodological approach (Figure 6.11). The initial step is identifying an environment of interest. Once placed in a sampling vessel, the entire community must be rapidly frozen at –80°C to keep the mRNA pool intact – so that it is indicative of in situ conditions ("RNA collection filter", Figure 6.11). Because the end goal of the protocol shown in Figure 6.11 was absolute quantification of discovered mRNAs, the source environmental sample was dosed ("spiked") with a known quantity of a known mRNA standard just after collection. The pool of nucleic acids is extracted from the sample. This is followed by removal of DNA (thorough DNase treatment), removal of rRNA (usually amounting to ~90% of total RNA; the rRNA is depleted via subtractive hybridization), amplifying the mRNA pool (sometimes an optional step), conversion of the mRNA to cDNA (single-strand cDNA synthesis), and then sequencing the cDNA pool (this may occur with or without a "second strand" cDNA synthesis step). Based on recovery efficiency of the mRNA molecules added to the environmental sample, the absolute numbers of detected community transcripts can be calculated (Figure 6.11).

Table 6.11 provides details from 4 metatranscriptomic investigations spanning seawater, soil, and human gut habitats. All used 454 pyrosequencing technology (though paired-end Illumina sequencing has also been successfully applied to metatranscriptomic inquiries). Large data sets (millions of reads) were uniformly generated – providing information about both highly expressed functional genes and the taxonomic identification of the hosts of the same genes. Entries 1 (seawater) and 3 (soil) in Table 6.11 utilized experimental designs that incubated habitat samples treated (respectively) with marine dissolved organic carbon or the model pollutant compound, phenanthrene. This approach to experimental design allows an unaltered "control" treatment to serve as the baseline, against which the paired response in the treatment is assessed. Entry 1 in Table 6.11 summarizes how McCarren et al. (2010) linked *Alteromonas*-related marine bacteria to the metabolism of marine-dissolved organic matter and stimulation of a food chain relying upon methylated compounds. Entry 2 in Table 6.11 reports detecting in situ expression of a variety of pathways influencing P- (e.g., phosphate uptake), N- (e.g., dissimilatory nitrate reduction), and S- (e.g., dimethylsulfoniopropionate cleavage) in marine waters (Gifford et al., 2011). Quantitative results stemming from the mRNA standard added to the extracted water sample indicated a shockingly low 0.00001% recovery (Gifford et al., 2011). Entry 3 in Table 6.11 showed that in soil the microbial community actively engaged in phenanthrene metabolism and oxygenases carrying out phenanthrene attack were enriched; the mRNA transcripts allowed de Menezes et al. (2012) to map changes in the abundances of at least 58-transcript-associated host cells (*Bacteria, Archaea*, fungi). Entry 4 in Table 6.11 discovered across 10 individual humans unexpected uniformity in the composition of families of metabolically active gut bacteria (dominated by 5 main groups) (Gosalbes et al., 2011).

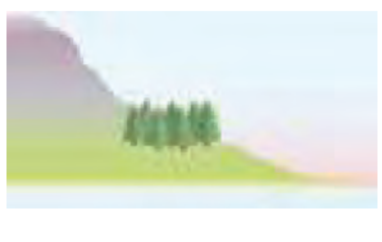
Table 6.11

Examples of next-generation metatranscriptomic investigations: their strategies, goals, and outcomes

Habitat sampled	Goal	Main sequencing technology	Accessory community composition assay	Number of samples	Total DNA sequenced	Outcome	References
1. Seawater (Hawaii)	Use community transcriptomics to discern genes involved in metabolism of dissolved organic carbon (DOC)	454 pyrosequencing of mRNA pool (from cDNA)	DNA pool also sequenced, flow cytometry cell sorting, 16S rRNA analysis	16 (4 sampling times, with and without added DOM, in duplicate)	~4.5 million reads	This 27-h incubation of seawater with and without added DOM showed rapid early response to DOC by *Alteromonas*-related bacteria, followed by a succession of microorganisms, especially those utilizing DOM conversion products: methyl sugars, methanol, and/or formaldehyde	McCarren et al., 2010
2. Coastal marine water, Southeastern, USA	Characterize transcriptomics of marine microbial community, quantify expression levels by including an internal standard mRNA template	454 pyrosequencing GS-FLX	None	2 replicate samples of seawater	2.1 million reads; 210 bp in length	Expression levels of 82 genes diagnostic for marine nitrogen, phosphorus, and sulfur transformation	Gifford et al., 2011

Table 6.11 *Continued*

Habitat sampled	Goal	Main sequencing technology	Accessory community composition assay	Number of samples	Total DNA sequenced	Outcome	References
3. Soil from wood treatment plant contaminated with polycyclic aromatic hydrocarbons	Obtain RNA-based view of how soil community responds to added phenanthrene	454 GS-FLX Titanium	None	4 soil samples (with and without phenanthrene in duplicate)	534,668 reads, 355 bp in length	Seventeen-day treatment of soil samples with and without added phenanthrene. As phenanthrene was metabolized, mRNA transcripts associated with aromatic-compound metabolism, stress response, and thioredoxin were boosted. Shifts in abundances of 58 types of *Bacteria*, *Archaea*, and fungi responding to phenanthrene were reported	de Menezes et al., 2012
4. Microbial community in human gut human fecal samples)	Characterize the transcriptome of human gut microbial community in 10 healthy individuals	454 pyrosequencing Titanium	None	10 healthy volunteers, 1 fecal sample from each	8.5 Gb, 489,307 reads, 124 bp in length	Key active taxa were *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceceae*, *Prevotellaceae*, and *Richenellaceae*. High functional expression of genes involved in carbohydrate metabolism, energy production and cell synthesis was found; amino acid and lipid metabolism were underrepresented	Gosalbes et al., 2011



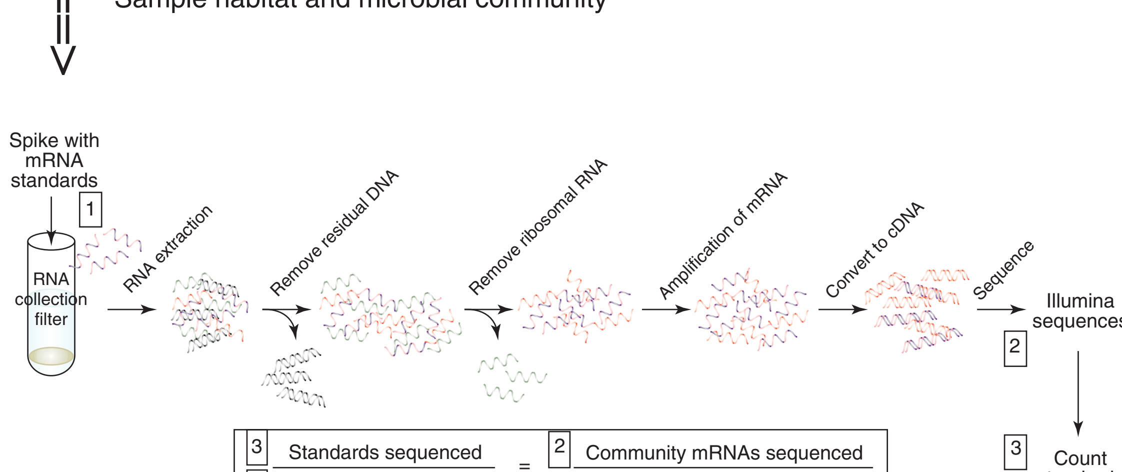


Figure 6.11 Environmental transcriptomics – listening to the metabolic messages delivered in naturally occurring microbial communites by the pool of expressed mRNA. Shown is a protocol for processing nucleic acids captured from an environmental sample such as ocean water. The total pool of nucleic acids (especially DNA + rRNA + mRNA) is processed in a manner that eliminates DNA (with DNase) and depletes the sample of rRNA, so that the signals from mRNA are enhanced. The protocol shown uses an internal standard (mRNA added in known amounts to the sampled community) to calculate average per-cell mRNA inventories. A known number of internal mRNA molecules are spiked into the environmental sample and the ratio of standards added to standards recovered in the high-throughput sequence library allows estimation of the numbers of natural mRNAs in the sampled community. (Figure by courtesy of M.A. Moran, Department of Marine Sciences, University of Georgia, with permission; see Gifford et al., 2011, detailed procedures.)

Microbial small RNA (sRNA) is a class of RNA, quite distinctive from tRNA, rRNA, and mRNA. In cell biology, microbial small RNA molecules have been recognized for their crucial role in posttranscriptional regulation of gene expression (Desnoyers et al., 2013). Typically sRNAs (ranging from 50 to 500 bp) target mRNAs. The impacts of mRNA binding by sRNA include alteration in mRNA stability and concomitant modulation of proteins produced by the translational apparatus. Shi et al. (2009) used metatransriptomic inquiry to characterize the diversity and abundance of sRNAs

in Pacific Ocean microbial communities at 4 depths near Hawaii. Results of this study provided insights into sRNA-based environmental sensing and response systems in natural microbial communities.

Next-generation metaproteomic investigations

The central dogma of molecular biology states that "DNA makes RNA makes protein". It follows, then, that *metaproteomics* is the natural complement to the above-described next-generation procedures characterizing microbial communities at the level of DNA (metagenome) and RNA (metatranscriptome). Constantly accruing bioinformatic data bases (e.g., NCBI, SwissProt, Genbank, Mass Spectrometry Sequence Data Base) have compiled sequence information about genes and their transcribed/translated products: proteins. This vast amount of information establishes an unprecedented opportunity for matching peptide fragments (chains of amino acids (peptides) created by digestion of proteins by the protease, trypsin, from unknown proteins in environmental samples to reference peptides in the established databases. Figure 6.12 displays the broad scheme of how metaproteomics works. Four steps take the investigator from a real-world field site of interest (sample gathering and preparation of a protein pool), to fractionation/analysis of the proteins present in the targeted microbial community (mass spectrometric data), to proteome bioinformatics (creating information identifying both proteins and the identity (phylogeny) of the cells hosting the proteins), to a final "data evaluation" phase that seeks independent validation/verification of the metaproteomic findings.

Though still in its infancy, environmental metaproteomics has immense promise for revolutionizing environmental microbiology (Hettich et al., 2012; VerBerkmoes et al., 2009; Schneider and Reidel, 2010). Much like phospholipid fatty acids (PLFAs; see Section 6.7), proteins are biomarker molecules present in population of cells dwelling in soils, sediments, and waters. Like PLFAs, protein pools can be directly extracted and they are not subject to quantitative distortion stemming from amplification procedures (e.g., PCR) routinely used for nucleic acids. Moreover, when identified, the matched protein simultaneously delivers information about both the function of the protein (in in situ metabolism – ranging from cell respiration to ATP synthase to sulfate reduction to nitrogen fixation) and the identity of the cell hosting the protein (the closest known phylogenetic match). Thus, in a single assay, metaproteomics delivers long lists (hundreds to thousands) of paired pieces of information about "who is there" *and* "what they are doing" (see Section 6.11).

- **Question:** Is metaproteomics the panacea that answers all the long-standing unanswered questions about "who is doing what" in environmental microbiology?
- **Answer:** Not yet, because of lingering methodological weaknesses.

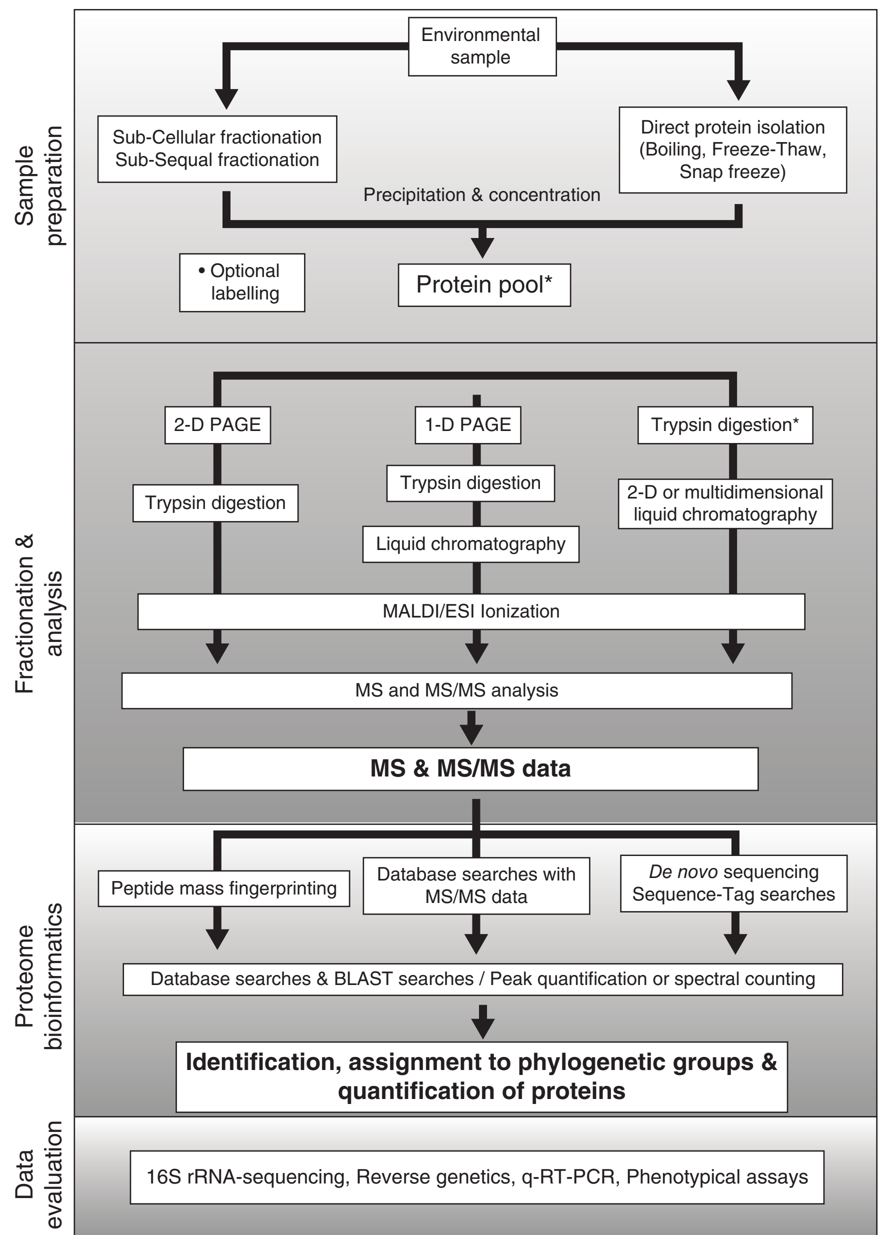


Figure 6.12 Environmental metaproteomics. Schematic flow chart depicting overview of environmental proteomics methodology: sample preparation, protein fractionation, preparation of peptides by digestion with trypsin, liquid chromatography/mass spectrometric analyses, database matching of peptide mass spectra, protein/identification, and optional quantification (indicated by *). Key final stages are proteome bioinformatics and data evaluation. Abbrevistions: PAGE, polyacrylamide gel electrophoresis; 2-D, two-dimensional; 1-D, one-dimensional; MALDI/ESI, Matrix Assisted Laser Desorption Ionisation–Mass Spectrometry/Electrospray Ionisation–Mass Spectrometry; MS/MS, tamdem mass spectrometry. (From Schneider, T. and K. Reidel. 2010. Environmental proteomics: analysis of structure and function of microbial communities. Proteomics 10:785–798, with permission from Wiley Publishing.)

Let us examine several of the key steps in Figure 6.12 to get an idea of current constraints on metaproteomic methods, and hence the resulting metaproteomic data. Obtaining protein pools requires that cells be gathered (from water on filters (facile), from soils and/or sediments (still a challenge)) and that cell lysis and separation from both cellular debris and environmental debris (detritus, humic substances) are effective and unbiased. Improvements in step 1 of Figure 6.12 are still needed. Regarding step 2 (fractionation and analysis), proteomic analyses have limited reproducibility, and overlap even between repeated injections of a single protein mixture. The tandem mass spectrometric (MS/MS) technique lying at the heart of data generation is extremely precise (ion fragments formed in the first mass spectrometric step are, themselves, subjected to additional fragmentation and precision mass measurement); this yields both the identity of the amino acids and their order (sequence) in the original peptide fragment. However, if complex mixtures of proteins (hundreds or thousands of proteins, some extremely abundant and some extremely rare) are analyzed, many can be missed. For this reason, fractionation is used to simplify the protein mixtures. Fractionation is achieved by polyacrylamide gel electrophoresis (PAGE, in 1 or 2 dimensions) and/or by liquid chromatography. Regarding step 3 in Figure 6.12, MASCOT, SEQEST, and X-tandem are the names of the widely utilized search engines that match mass spectrometric data from the unknown peptides in environmental samples to peptides and their parent proteins in databases containing primary reference sequences. The algorithms for assuring accuracy of matches and assessing false discovery rates (false-positive identifications) are extremely powerful. However, the calls made by the search engines are limited by our current knowledge of known proteins, genes, and hosts. As knowledge of microbial diversity (and accompanying genetic and protein-functional diversity) advances, so also will advance our confidence in the ability of metaproteomics to accurately identify protein metabolic function and the identity of the cell's hosting environmental proteins. There are a variety of strategies for developing confidence that the matches between environmental peptides and their identified function and host are accurate. Redundancy is one strategy: little credence is given to a single match between a given protein in the database and the mass of an unknown environmental peptide; but if the metaproteomic data set being scrutinized contains 2 or more peptides that map the same reference protein, accuracy of the identification is assured. Another major consideration is if a metagenomic "blueprint" of the microbial community of interest has been established prior to the metaproteomic survey. If not, the peptide masses can be matched to known proteins or to *in silico* predictions of peptides encoded by genes in DNA-sequence data banks. However, the mapping of metaproteomic data from a microbial community on to a metagenome of the same community (community "proteogenomics") yields a higher level of protein identification (Hettich et al., 2012; VerBerkmoes et al., 2009). Care must be used when interpreting metaproteomic data: there is a strong impulse for the

investigator to assume that rates of in situ microbial metabolic processes are likely to be proportional to the sizes of protein pools involved in the biogeochemical processes under scrutiny. However, Moran et al. (2013) point out that protein abundances can easily become uncoupled from the fully integrated physiological processes (e.g., photosynthesis, sulfate reduction, nitrification, denitrification) to which the proteins contribute. Such uncoupling may be rooted in several mechanisms:

(i) Rates of enzymatic reactions are strongly influenced by both the concentration of substrates being transformed and by possible posttranslational modification (regulation) of the protein.

(ii) Many enzymes are constitutively expressed – as such, they are unlinked to the process of interest.

(iii) Induced proteins may outlive the resources they were synthesized to exploit.

(iv) Some proteins synthesized in response to scarcity may be most abundant when physiological reaction rates are at their lowest level.

Despite the list of caveats stated above, insights provided by metaproteomic analyses of microbial communities in complex naturally occurring habitats can be significant – yielding robust and often unprecedented information about ecologically important microorganisms and the processes they carry out in their native habitats. Table 6.12 provides an overview of four prominent metaproteomic investigations. Interest in the composition and metabolism of marine communities extending from the coast of Africa – west toward South America – led Morris et al. (2010; entry 1, Table 6.12) to complete a metaproteomic investigation. Many physiologically activity marine *Bacteria* were identified along the gradient from high nutrients to low nutrients; furthermore, the archaeal protein for ammonia monooxygenase was consistently found in the coastal waters – indicating the role of *Archaea* in in situ nitrogen cycling (Morris et al., 2010). Entry 2 in Table 6.12 summarizes results of a metaproteomic study examining carbon cycling in the litter layer of forest soils. Schneider et al. (2012) found that fungi were the main producers of extracellular hydrolytic enzymes involved in plant-organic matter decomposition; the 6 main active fungal phyla were identified, as was a seasonal shift in activities (from February to May). Kolmeder et al. (2012; entry 3, Table 6.12) used metaproteomics to gain insights into the human gut microbiome of 3 healthy humans over a period of 6 to 12 months. Dominant microbial transformations in the gut were anaerobic – involved with carbohydrate uptake/transformation, glycolysis, gluconeogenesis, pyruvate metabolism, and, especially, glutamate dehydrogenase; metaproteomes were stable over time and host-specific (Kolmeder et al., 2012). The final entry (4, Williams et al., 2012) in Table 6.12 reports contrasts between winter and summer of microbial hosts and their metabolic processes in marine waters near Antarctica; these authors were able to identify a large array of microbes active in situ, to link them to S, N, and C cycling, and to portray shifts in community metabolism between summer and winter.

Table 6.12

Examples of next-generation metaproteomic investigations: their strategies, goals, and outcomes

Habitat sampled	Goal	Main analysis technology	Accessory assays	Number of samples	Total protein spectra	Outcome	References
1. South Atlantic ocean along a gradient extending from nutrient upwelling in coastal Africa, west across the low nutrient Atlantic gyre	Apply comparative metaproteomics to access community composition and function	LC-MS/MS LTQ-Orbitrap mass spectrometry	Ocean geochemistry of the sampled sites; 16S rRNA clone libraries	10	5389 peptides, 2273distinct proteins	Membrane-enriched cell fraction was used to generate peptide spectra. Most frequently identified proteins were involved in membrane transport (nutrient uptake), proteins of unknown function, outer membrane proteins. Virus proteins were ubiquitous. Many taxa (e.g., *Cyanobacteria*, *Betaproteobacteria*, *Crenarchaeota*) shifted abundance from coastal to mid-Atlantic habitats. Rhodopsin proteins (light-driven protein pumps) were found and their hosts identified	Morris et al., 2010
2. Four beech-forest soil sites in Austria	Link structure and function of leaf-litter-degrading microbial communities and tie this to seasonality and nutrient content	1D-SDS-PAGE and LC-MS/MS; LTQ-Orbitrap mass spectrometry	Leaf-litter nutrient content, PLFA community characterization, cellulose and chitinase enzyme assays	8	8895 proteins; 1724 unique protein clusters	Taxonomic identification of host of proteins showed 62% *Bacteria*, 17% metazoa (animals), 8% fungi, and 6% plants. Fungi were the dominant producers of litter-degrading enzymes – especially phosphatases, cellulases, pectinases, xylanases, lipases, and chitinases. The abundance of litter-degrading enzymes varied with forest sampling sites	Schneider et al., 2012

3. Human intestinal microbiota in 3 healthy adults for 6–12 months	Examine the composition and temporal stability of intestinal microflora	1D-SDS-PAGE and LC-MS/MS, LTQ orbitrap mass spectrometry	16S rRNA hybridzation assay using Human Intestinal Tract microarray	37	~100,000 peptide specra identifying 1790 proteins	Most common core protein was glutamate dehydrogenase associated with *Lachnospiracea*, *Bacteroidaceae*, *Ruminococcoceae*, and *Bifidobacteraceae* Human fecal proteome is subject-specific and stable over a 1-year interval, a stable common core of ~1000 carbohydrate-transport and -degrading proteins appears widespread	Kolmeder et al., 2012
4. Antarctic peninsula Southern Ocean surface waters	Use metaproteomics to contrast metabolism and composition of Antarctic marine waters in summer versus winter	1D-SDS-PAGE; LC MS/MS Orbitrap mass spectrometry	Physical/Chemical (geochemistry, salinity, chlorophyl; temperature) of ocean waters	9 (6 in summer; 3 in winter)	1061 proteins	Seventy-four physiological/biogeochemical proteins were mapped on to ~10 key bacterial taxa (e.g., *SARII*, *Roseobacter*, *Sphingomonadales*, *Bacteroidetes*, *Nitrosopumilus*, Marine Group I *Crenarchaoeta*). Metabolic community characteristics were depicted among autotrophs and heterotrophs for both summer and winter	Williams et al., 2012

LC = liquid chromatography; SDS = sodium dodecyl sulfate; PAGE = polyacrylamide gel electrophoresis; MS = mass spectrometry; MS/MS = tandem mass spectrometry (see text); PLFA = phospholipid fatty acid-based determination of community composition.

Omics-enabled large-scale surveys of global microbial diversity

The omics methodologies, especially the new DNA sequencing technologies described above, generate vast individual data sets that capture the genetic heritage of microbial communities that have dominated our biosphere for billions of years. If we admit that the "great unknown" in environmental microbiology is the extent of microbial diversity on our planet, why not confront the issue directly?

- **Question:** Are omics technologies (especially DNA sequencing coupled to bioinformatics) the right tools for tackling the job of describing the biosphere's complete microbial diversity?
- **Answer:** The prospects look excellent.

Several international consortia (groups of cooperating scientists, organizations, institutions) have proposed implementation of systematic large-scale exploratory efforts aimed at discovering the extent of our planet's microbial diversity. One such effort is the Earth Microbiome Project (EMP; Gilbert et al., 2010, 2014); another is the Terra Genome Project (Vogel et al., 2009). There are approximately 10^{30} microbial cells on Earth (see Section 4.5). According to Gilbert et al. (2010), the average quantity of DNA in each of these cells is ~10 million base pairs. Yet to date the total global environmental DNA sequencing effort has produced <1% of the total DNA found in a liter of seawater or a gram of soil (Gilbert et al., 2010, 2014). To quote the vision statement of the Earth Microbiome Project: "Hence, we have vastly under-sampled the complexity and diversity of microbial life on Earth. Recent advances in high-throughput sequencing technologies have provided an unprecedented opportunity to explore the microbial universe" (Gilbert et al., 2010, 2014).

> The Earth Microbiome Project presents a revolution in how we tackle the challenge of understanding the interactions among microbes and their environments, and defines both questions and a potential suite of tools to provide answers. We wish to sequence microbes and microbial communities from a broad range of biomes (an environment with unique environmental parameters; e.g., a hydrothermal vent on an abyssal plain) to achieve three main goals. First, to define microbial community structure, and to explore the factors that affect community structure at different scales. Second, to explore the protein universe and attempt to produce a complete inventory of protein family diversity. Finally, to curate this information to create a global database of samples, genes and proteins that can be used to answer fundamental questions about the ecology of life on and off the earth...... We will analyze these communities using metagenomics, metatranscriptomics and amplicon sequencing to produce a global Gene Atlas describing protein space, environmental metabolic models for each biome, approximately 500,000 microbial genomes, and a data-analysis portal for visualization of all information (Gilbert et al., 2010).

Stay tuned!

6.11 DISCOVERING THE ORGANISMS RESPONSIBLE FOR PARTICULAR ECOLOGICAL PROCESSES: LINKING IDENTITY WITH ACTIVITY

It is appropriate, at this point, to recall the remarks from various scholars shown in Table 6.1 about methodological limitations in environment microbiology. Given the independent challenges of answering "Who is there?" and "What are they doing?" it may seem glaringly optimistic to ask "Can the microorganisms responsible for particular biogeochemical processes in the field be identified?" After all, procedures for documenting in situ biogeochemical activity (without discerning the particular responsible party) are rather demanding (see Sections 6.4 and 6.8). Thus, studies pursuing the goal of identifying the microorganisms responsible for particular biogeochemical field processes are ambitious. Yet, they are crucial for advancing environmental microbiology and improving our ability to manage the biogeochemical processes that maintain the biosphere. Linking a microorganism's identity to its activity in a given habitat has traditionally proven to be an evasive goal. There are several prominent exceptions to this rule (see below and later in Table 6.14). Very significantly, the long-standing void (information gap) separating "Who is there?" from "What are they doing?" in environmental microbiology is rapidly being filled by new information via methodological innovations discussed below (e.g., Stable Isotope Probing) and in Sections 5.10 (biogeography) and 6.10 (omics methodologies). The connection between in situ identity and in situ activity forged by metatranscriptomics and metaproteomics (see Section 6.10 and below) promise to establish landmark inventories documenting the identities of key players in microbial biogeochemical processes across hundreds, or thousands, of habitats.

This section presents a perspective on past and current attempts to discover the identity of microorganisms that are responsible for catalyzing the key biogeochemical reactions that occur in situ in soils, sediments, and waters. Insights are sought by contrasting ways of documenting causality in medical microbiology – Koch's postulates – with those of environmental microbiology. We will rely upon Figure 6.13, which presents a model for the generation and interpretation of environmental microbiological information that integrates five key considerations: (i) complexity of the experimental system under study; (ii) the path of inquiry taken by investigators to generate information; (iii) methodologies; (iv) data/information generated by the methods; and (v) procedures to assure ecological validity of the data.

Koch's postulates in medical microbiology and environmental microbiology

In 1884, Robert Koch developed fundamental criteria for proving that a particular microorganism (*Bacillus anthracis*) was responsible for a particular

process (anthrax disease) in a particular habitat (sheep). This generalized four-step guideline, known as Koch's postulates, is as follows:

1. The microorganism should be found in all cases of the disease in question, and the microorganism's distribution in the body should be in accordance with the lesions observed.
2. The microorganism should be grown in pure culture *in vitro* (or outside the body of the host) for several generations.
3. When such a pure culture is inoculated into susceptible animal species, the typical disease must result.
4. The microorganism must again be isolated from the lesions of such experimentally produced disease.

Koch's postulates have been the gold standard in medical microbiology for establishing causality and have survived intact to the present, with minor modifications that accommodate recent molecular biological techniques (Brooks et al., 2010; Falkow, 2004). The fifth column in Figure 6.13's paradigm provides a schematic mechanism for ecological validation of data, including inoculation to verify Koch's postulates (IVKP). Below are suggestions why medical microbiologists have traditionally been far more successful than environmental microbiologists in identifying causative agents.

Table 6.13 compares and contrasts for medical and environmental microbiology four key factors that influence the determination of causality: complexity of the habitat plus its inhabitants, the process of interest, identifying a potential agent, and linking the agent to actual field processes. As stated in Table 6.13, human disease is readily recognized in the field (afflicted humans in society convey contagious agents) and has an enormous detrimental impact. Thus, the impetus for understanding and intervening is also enormous. In contrast, the impetus for the discovery and management of ecologically important biogeochemical reactions has been less pressing – perhaps because biogeochemical processes in field habitats are not facile to discern and because such processes generally proceed regardless of intervention.

"Culturability" is the other major factor that has likely allowed medical microbiology to flourish while environmental microbiologists have perhaps fallen out of step. Culturability is a direct reflection of two interacting issues: (i) the relative ratio of target to nontarget organisms in the initial inoculum and (ii) an ability to accurately simulate the native habitat in media. When Robert Koch embarked down the cultivation-based path (Figure 6.13, column 2), his initial field sample (blood from a diseased sheep) was essentially a monoculture containing a "large number of regular, rod-shaped, colorless, immotile structures" (Koch, 1884) that were microscopically discernible. Compare this to the vast, confusing zoo of candidates (e.g., thousands of species and 10^9 cells per gram of soil) that confront a soil microbiologist. Furthermore, Robert Koch found that the blood-borne bacilli readily reproduced on solid media containing "nutrient gelatin or boiled potato" (Koch, 1884). Facile culturability is not a given in

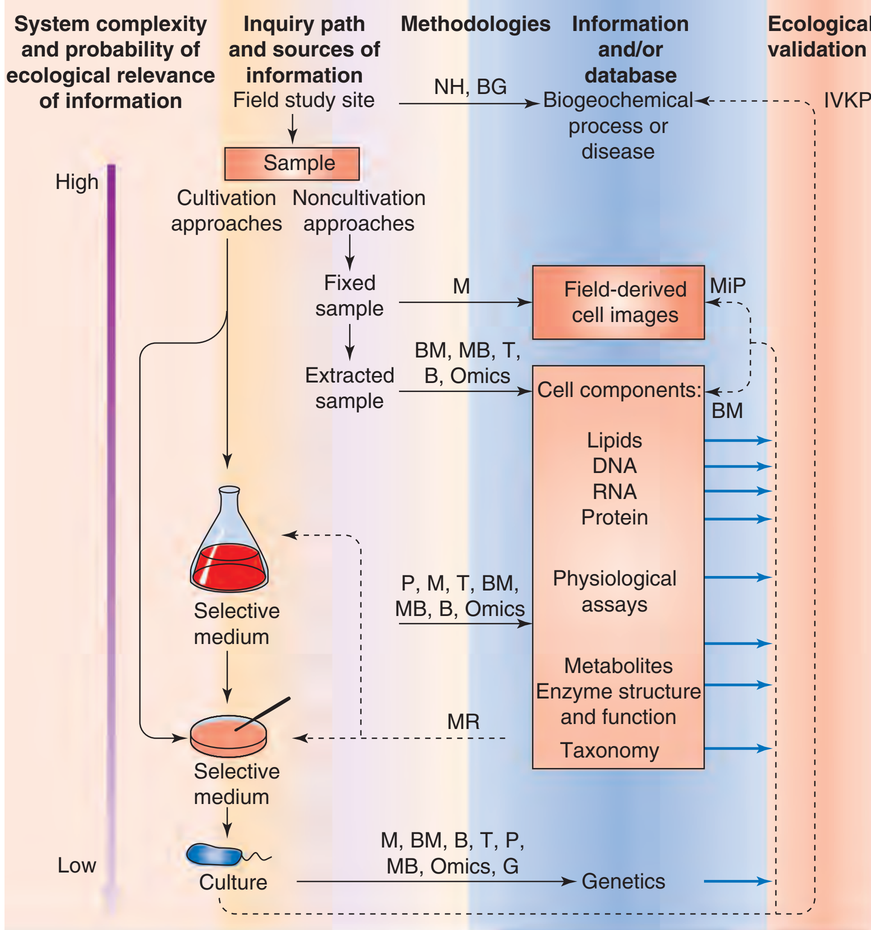


Figure 6.13 Model for the generation and interpretation of environmental microbiological information, with emphasis on field relevance and ecological validation of data. Column 1 provides a scale for evaluating the likely ecological relevance of information in the other four columns. With each successive methodological step away (down) from direct field measurements, the risk of artifacts (ecologically misleading data) increases. Column 2 provides an outline of microbiological procedures (cultivation-based or nonculture-based) that are used as sources of information about microorganisms in nature. Column 4 shows the types of information created by various methodological procedures (column 3). The dashed arrows in column 5 show the main feedback pathways that can be used to validate the ecological relevance of microbiological data. Dashed arrows connecting column 4 to column 2 show a means for improving growth media, as guided by field-derived "omics" information. B, biochemical characterization, BG, biogeochemistry; BM, biomarkers (e.g., 16S rRNA genes, lipids); G, genetic characterization (e.g., operons, regulation); IVKP, inoculation to verify Koch's postulates; M, microscopy; MB, molecular biological characterization (e.g., cloning and sequencing); MIP, microscopic probing (immuno- and omics-based visualization in field-fixed cells); MR, medium refinement based on expressed genes and other biomarkers discovered in field samples; NH, natural history; Omics, genomics, proteomics, metabolomics, transcriptomics, and so on; P, physiological characterization; T, taxonomic characterization. (Reproduced and modified with permission from *Nature Reviews Microbiology*, from Madsen, E.L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Rev. Microbiol.* **3**:439–446. Macmillan Magazines Ltd, www.nature.com/reviews.)

Table 6.13

Contrasts between information on causality in medical and environmental microbiology. (Reproduced and modified with permission from *Nature Reviews Microbiology*, from Madsen, E.L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Rev. Microbiol*. **3**:439–446. Macmillan Magazines Ltd, www.nature.com/reviews)

Medical microbiology	Environmental microbiology
Traits of habitats studied	
Habitat is human body Globally distributed Evolutionarily stable	Soils, sediments, and waters are globally distributed, but show high physical and geochemical variability in time and space
Consistent uniform resources for microbial colonization	Highly variable resources; severe but unpredictable nutritional limitation is the rule
Reliably simulated in laboratory media or animal model	Unreliably simulated in the laboratory because geochemical complexity defies characterization
Low-diversity microbial community offers few background organisms that confound isolation of causative agent	Extremely high community diversity; thousands of background organisms that can be mistaken for causative agents
Characteristics of microbial processes	
Diseases are reliably recognized in the field Immense negative impact on host; intervention essential	Biogeochemical reactions are often difficult to document in the field; geochemical footprints of processes may not be apparent in open field sites
Huge impetus for scientific study (disease prevention)	Robust, reliable processes often have positive impacts, regardless of human understanding or intervention Historically, little impetus for scientific study, relative to human disease
Steps to identify potential causative agents	
Pathogens are often culturable because habitats (hosts) are well simulated in laboratory media or animal model	Biogeochemical agents often have not yet been cultured because habitats are so poorly understood, so difficult to simulate
Disease specificity and habitat uniformity assure that a single agent is the cause of global problem	Large- and small-scale habitat diversity may select for many different agents within flexible ecological guilds that carry out processes
Relatively high chance of isolating correct organism because it comes from low-diversity community	Relatively low chance of isolating ecologically significant agents because community diversity is immense Process may stem from many cooperating populations
Ways of linking identity to field processes	
Koch's postulates are well established for medical microbiology	Owing to habitat complexity, community complexity, culturing challenges, and functional redundancy in communities, Koch's postulates rarely apply Topic of ongoing multidisciplinary research involving microscopy, biomarker probes, stable isotopic signatures, autoradiography, stable isotopic probing, metaproteomics, metatranscriptomics, and other strategies

medical microbiology (e.g., *Treponema pallidium* (syphilis) and *Mycobacterium leprae* (leprosy) cannot yet be grown *in vitro*; Brooks et al., 2010), but uniform, stable, globally distributed nutrient conditions of the human body are undeniably easy to mimic in growth media relative to the uncharacterized, site-specific, heterogeneous complexity of soils, sediments, and waters (see, for example, Sections 3.6, 4.6, and 6.2 and Box 6.1). Many biogeochemical processes are not catalyzed by individual microorganisms, but instead by cooperating populations (consortia). Moreover, it seems likely that guilds of physiologically equivalent microorganisms in different habitats may be compositionally distinctive (Table 6.13).

Thus, identifying ecologically significant microorganisms using Koch's postulates has been evasive because of a combination of impetus, community complexity, and limitations of cultivation techniques. Fortunately, other paths toward ecological validation exist that do not require cultivation of microorganisms. These paths (horizontal arrows in column 5 of Figure 6.13; Table 6.13, lower right) often rely upon microscopic probing of field-fixed cell images for DNA, RNA, or other biomarkers indicative of cell identity and/or activity (see below).

Linking field biogeochemical processes to responsible agents

Progress has been made on many fronts that contribute to successful identification of ecologically significant microorganisms. These fronts include impetus for inquiry, deciphering community complexity, improving cultivation procedures, as well as development of new strategies and techniques that largely substitute for Koch's postulates (during the interim while microorganisms in biosphere habitats remain uncultured). Substitutes for Koch's postulates are outlined in the fifth column, bottom row of Table 6.13, and detailed examples are discussed below, especially as presented in Table 6.14.

Increasing impetus for understanding microbially mediated environmental processes probably reflects the growing public and governmental awareness of the frailty of our planet (e.g., Raven, 2002; Sugden et al., 2003; Tollefson and Gilbert, 2012; Ehrlich et al., 2012; Zencey, 2010; Biermann et al., 2012) under stresses of population growth, climate change, pollution, and disease transmission. Understanding the complexity of habitats and naturally occurring microbial communities is implicit in current research areas and exemplified by geochemical characterization of ocean hydrothermal vents (Reysenbach and Shock, 2002), Lake Vostok buried deep beneath polar ice (Jouzel et al., 1999; Inman, 2005), and also by recent metagenomic whole community genome-sequencing efforts (Hallam et al., 2004; Tyson et al., 2004; Venter et al., 2004; Rusch et al., 2007; Varin et al., 2012; Ferrer et al., 2013; Lewin et al., 2013; Delmont et al., 2012; Denef and Banfield, 2012; Iverson et al., 2012; Hess et al., 2011; Wrighton et al., 2012; Albertsen et al., 2013; Human Microbiome Project, 2012). Cultivation strategies have already taken a significant leap forward

Table 6.14

Selected examples of efforts in environmental microbiology to identify microorganisms responsible for field biogeochemical processes. (Reproduced and modified with permission from *Nature Reviews Microbiology*, from Madsen, E.L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Rev. Microbiol.* **3**:439–446. Macmillan Magazines Ltd, www.nature.com/reviews)

Process	Microorganism	Setting	Strategy	Commentary	References
1. Nitrogen fixation	*Rhizobium*	Nodule on root of legume plant in field	Inoculate soil lacking native *Rhizobia*	Success for Koch's postulates. Root infection process selects for the bacterial symbiont. Nitrogen fixation results from inoculation and nodulation	Fred et al., 1932
2. Biodegradation of trichloroethene (TCE)	*Dehalococcoides*	Groundwater beneath Air Force base	Inoculate subsurface habitat where TCE persists	A version of Koch's postulates succeeds. Metabolism only occurred after inoculation	Major et al., 2002
3. Glutamate uptake and DNA synthesis	Unknown	Water samples from Long Island Sound and Narragansett Bay	Microbiology combined with microautoradiography; incubated in the laboratory	First attempt to capture microscopic image of cells incorporating radiolabeled compounds. A highly contrived laboratory setting was required for use of radiolabeled substrates (^{14}C glutamate and tritiated thymidine)	Brock and Brock, 1966
4. Nitrification (and $^{14}CO_2$ fixation)	*Nitrobacter* (autotroph)	Sediment samples from Mammoth Cave, KY, enriched on nitrate incubated in the laboratory	Microscopy combined with microautoradiography and immunofluorescent detection of cells	First attempt to apply both fluorescent antibodies (identity) and autoradiography (activity) to soil microorganisms; highly contrived laboratory setting required for use of radiolabeled substrate ($^{14}CO_2$); $^{14}CO_2$ incubation did not directly assay nitrification	Fliermans and Schmidt, 1975
5. Nitrification (and $^{14}CO_2$ fixation)	*Nitrosococcus*, *Nitrosomonas* (autotrophs)	Seawater in bottles incubated aboard ship	Microscopy combined with microautoradiography and immunofluorescent detection of cells	Incubation in bottles with radioactive $NaHCO_3$ was brief under "field-like conditions"; two known nitrifiers were probed with fluorescent antibodies; $^{14}CO_2$ incorporation did not directly assay nitrification	Ward, 1984

6. Amino acid assimilation, DNA synthesis	Alphaproteobacteria, *Cytophaga-Flavobacterium* group	Coastal California seawater samples	Microscopy combined with microautoradiography and 16S rRNA FISH detection of cells	Samples (40 ml) of seawater were incubated in the laboratory for 3 h. Uptake of added tritiated glucose and amino acids was measured and imaged via autoradiography. 16S rRNA-based FISH identified active cells	Ouverney and Fuhrman, 1999
7. Organic and inorganic nutrient assimilation	Beta-Proteobacteria	Activated sewage sludge samples	Microscopy combined with microautoradiography and 16S rRNA FISH detection of cells	Samples (2 ml) of sewage sludge were incubated in the laboratory for 2–3 h. Uptake of added ^{14}C acetate, butyrate, bicarbonate, and ^{32}P-phosphate were measured and imaged via autoradiography. 16S rRNA-based FISH identified active cells	Lee et al., 1999
8. Glucose and acetate assimilation	Candidatus *Meganema perideroedes*	Activated sewage sludge samples	Microscopy combined with quantitative microautoradiography and 16S rRNA FISH detection of cells	Samples (2 ml) of sewage sludge were harvested the day before and kept at 4 °C, and then experiments were incubated in the laboratory for 1 h at 21 °C. Uptake of added ^{14}C acetate and glucose were measured and imaged via autoradiography. 16S rRNA-based FISH identified active cells	Nielsen et al., 2003
9. Methane oxidation	*Methylomonas*	Baltic Sea sediment sample	Simultaneous FISH probing of cellular rRNA (identity) and expression of methane monooxygenase expression (mRNA)	Demonstrated principle of using fixed samples for determining both identity and activity. The probed community had been enriched on methane and incubated in the laboratory for 4 weeks. Methane oxidation was not geochemically confirmed	Pernthaler and Amann, 2004

Table 6.14 ***Continued***

Process	Microorganism	Setting	Strategy	Commentary	References
10. Anaerobic methane oxidation	*Archaea* and sulfate reducers	Ocean sediments adjacent to methane sources (Pacific and Black Sea)	Follow stable isotopic signature of the ^{13}C methane into community biomarkers, cells, and site carbonate deposits	All biomarker, microscopy, and geochemical assays were performed on field-fixed samples. Resultant data support a single explanation; methane is oxidized anaerobically by a consortium of bacteria related to methanogens and sulfate reducers	Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001; Michaelis et al., 2002
11. Assimilation of acetate and methane	*Desulfotomaculum acetoxidans*, type I methanotrophs	Samples of sediments from Tamar mud flat and Lake Loosdrecht	Stable isotopic probing, following ^{13}C-labeled substrates into lipid biomarkers	Small sediment cores incubated in the laboratory for 8 h (acetate) and 14 days (methane). Polar lipid-derived fatty acids were extracted and analyzed by gas chromatography/isotope ratio mass spectrometry	Boschker et al., 1998
12. Assimilation of methanol	Alpha-Proteobacteria, *Acidobacterium*	Sample of oak forest soil	Stable isotopic probing, following ^{13}C-labeled substrate into DNA	Sieved, air-dried soil (10 g samples) fed ^{13}C methanol at 0.5% concentration for 44 days. DNA was then extracted, separated by ultracentrifugation, and the ^{13}C DNA fraction was sequenced and analyzed for 16S rDNA sequences	Radajewski et al., 2000
13. Phenol biodegradation	*Thauera*	Sample from laboratory bioreactor	RNA stable isotope probing, ^{13}C-labeled RNA was extracted, reverse transcribed, and sequenced	First demonstration that the RNA pool can be rapidly labeled. ^{13}C atoms were traced into the ribosome fraction of the community within 24–72 h. Sequencing of reverse transcribed RNA revealed identity of active microbes	Manefield et al., 2002

14. Methanol oxidation by denitrifying microorganisms	*Methylobacillus*, *Methylophilus*	Sample from laboratory bioreactor	DNA stable isotope probing, confirmed by both FISH and microautoradiography	^{13}C atoms were traced into a DNA clone library during a 24 h incubation. 16S rDNA sequences in library were confirmed by FISH; these, in turn, were confirmed by microautoradiography using radioactive methanol	Ginige et al., 2004
15. Naphthalene biodegradation	*Polaromonas naphthalenivorans*	Contaminated field sediment in South Glens Falls, NY	Field-based DNA stable isotope probing	Addition of ^{13}C naphthalene to field site sediment; respiration assay confirmed in situ biodegradation; extraction and sequencing of the 16S rRNA genes in ^{13}C DNA identified the responsible population. A representative of the population was cultured	Jeon et al., 2003
16. Uranium reduction and immobilization	*Geobacter*	Contaminated subsurface sediment in Rifle, CO	Addition of electron donor (^{13}C and ^{12}C acetate) to field for stimulation of U(VI) reduction; molecular biomarker analyses and field stable isotope probing	Weight of evidence shows very strong association between stimulated U(VI) reduction and an increase in *Geobacter* biomarkers	Anderson et al., 2003; Chang et al., 2005
17. Anaerobic ammonia oxidation (anammox)	Candidatus *Kuenenia stuttgartiensis*	Anaeobic biorector and marine sediments (Costa Rica, Black Sea, Africa)	Document biomarkers in field samples (unique ladderane lipids, 16S rRNA sequences via cloning and microscopy) and incubations of field samples with $^{15}NH_4$ followed by mass spectrometric analysis of the resulting N_2	The case is very convincing: combinations of anammox-specific biomarkers, FISH microscopy, and a physiological assay that distinguishes N_2 production via nitrate reduction versus ammonia oxidation	Dalsgaard et al., 2003; Kuypers et al., 2003, 2005; Schmid et al., 2005

Table 6.14 *Continued*

Process	Microorganism	Setting	Strategy	Commentary	References
18. Nitrogen fixation	*Trichodesmium*	Ocean water	Recognizable filamentous colonies were collected by filtration and assayed, physiologically, in bottles aboard ship	Weight of evidence leaves no doubt that *Trichodesmium* fixes nitrogen in ocean waters; nitrogen fixation assays relied upon closed-bottle incubations; field activity has been confirmed by immunodetection of nitrogenase enzyme in field samples	Paerl et al., 1989; Capone et al., 1997; Montoya et al., 2004
19. Photosynthesis	*Prochlorococcus*	Ocean water	Rates of cell division and photosynthesis were inferred from circadian cell cycles at many depths	Field-fixed cells were analyzed by flow cytometry; specific growth rate was estimated by analyzing circadian cell cycle patterns; CO_2 fixation was implicit in growth of this photosynthetic microorganisms	Vaulot et al., 1995
20. Metabolism of naphthalene in seawater	*Alteromonas*	Oil-contaminated marine coastal sediments, Korea	Two-year field study quantitatively monitoring abundances of 16S rRNA genes and mRNA transcripts of naphthalene dioxygenase	A strain of *Alteromonas* growing on polycyclic aromatic hydrocarbons was isolated and its 16S rRNA and naphthalene dioxygenase genes were found to be abundant in situ	Jin et al., 2012
21. Nitrite oxidation, amino acid transport, carbon cycling	*Nitrospirae*, *Alteromonas*, euryarchaeal Marine Group II, respectitvely	Gulf of Mexico, deep ocean, Guaymas basin	Shot-gun sequencing (Illumina HiSeq 2000) of cDNA prepared from RNA originating in 6 water samples	Metatranscriptomics of several ecologically important genes were mapped to their hosts	Baker et al., 2013
22. Oxidation of ammonia, methane, and sulfur	*Nitrosopumilus*/Marine group I *Archaea*, *Methylobacter*/ *Methylomonas*/ *Methylomicrobium*, and SUP05 *gammaproteobacteria*, *respectively*	Plumes of water emerging from hydrothermal vents, Guaymas Basin	454 pyrosequencing of cDNA prepared from RNA originating in 6 samples	Metatranscriptomics allowed mapping of ecologically expressed genes to their hosts	Lesniewski et al., 2012

23. Aerobic ammonia oxidation, anamox (other processes)	*Nitrosopumilus maritimus*, *Kuenenia stuttgartensis*, respectively (also *Pelagibacter* and SUP05)	Eastern Tropical South Pacific oxygen minimum zone	454 pyrosequencing of cDNA prepared from RNA originating in 4 sampling depths	Metatranscriptomics allowed mapping of expressed genes to their hosts	Stewart et al., 2012
24. Aerobic ammonia oxidation, nitrite oxidation, carbon fixation, nutrient uptake, taurine degradation, glycine betaine degradation, methylotrophy	SAR11, *Roseobacter*, *Planctomyces*, *Sphingomonadales*, *Bacteroides*, *Nitrosopumilus*, Marine Group I, *Crenarchaeota*	Southern Ocean, Antarctic Coastal Water	ID-SDS PAGE; LC-MS/MS analyses of community proteins	Metaproteomics allowed identification of both biogeochemical processes and microbial hosts of proteins; 9 water samples	Williams et al., 2012
25. Cellulose decomposition (and other processes)	Five key fungi were hosts of cellulose enzymes; *Dothideomycetes*, *Leotiomycetes*, *Agaricomycetes*, *Sordariomycetes*, and *Eurotiomycetes*	Litter layer in 4 beech forest soils in Austria	ID-SDS PAGE; LC-MS/MS analysis of community proteins	Metaproteomics allowed identification of both biogeochemical processes and microbial hosts of proteins; 8 samples	Schneider et al., 2012

FISH, fluorescent in situ hybridization.

via efforts in which minimally altered environmental samples are used to meet the complex and subtle nutritional needs of naturally occurring microorganisms (Button et al., 1993; Kaeberlein et al., 2002; Rappé et al., 2002; Zinder, 2002; Leadbetter, 2003; Nichols et al., 2010; Vartoukian et al., 2010; Zengeler et al., 2007; Zengeler, 2008; see also Sections 6.2 and 6.6).

Using Figure 6.13 as a map for visualizing steps toward progress in environmental microbiology, there are three obvious avenues for increasing the ecological validity of information. First, if the media used in the flask and Petri dish assays (cultivation-based inquiry path, column 2, Figure 6.13) improve in ecological relevance, then the microorganisms eventually isolated are far more likely to be active in nature (the culture will fall closer to the top of column 1). Second, as analyses of field-fixed extracted samples (column 4) deliver increasingly sophisticated information about expressed genes and proteins used by microorganisms in their native habitats, inferences can be made about ambient physiological conditions, carbon substrates, and nutritional needs. Such information can guide the design of media so that new organisms can be cultured. Finally, the several paths of information flow (column 5) for validating data need to be thoroughly utilized. These validation paths are: (i) following Koch's postulates by the addition of cultures to field sites; (ii) the use of pure culture-derived "omics"-based biomarkers to guide analyses of extracted samples; and (iii) using microscopy and biomarker probes to confirm field relevance of information from both pure cultures and extracted samples.

Selected examples of past and current investigations aimed at linking identity of microorganisms to their field activity are shown in Table 6.14 (a glossary for technical terminology is presented in Table 6.4). Entries in Table 6.14 were chosen to be representative of the types of strategies, techniques, challenges, and breakthroughs that have occurred in environmental microbiology over the last several decades. Emphasis is upon identifying microorganisms and being sure their biogeochemical reactions were catalyzed in situ – in real-world field sites containing soil, sediment, or water. The first two entries (symbiotic nitrogen fixation and biodegradation of trichloroethene in contaminated groundwater) reveal that medical microbiology's paradigm (Koch's postulates) can be powerful and insightful. Koch's postulates are only applicable in limited contexts because the active microorganisms must be cultured and initially be in low numbers or absent from the inoculated habitat. The next six entries (3–8) in Table 6.14 illustrate the foundations and later developments in microscopy-based attempts to link identity to activity without using Koch's postulates. Microscopy and microautoradiography were initially used to see which cells in mixed microbial communities incorporated radiolabeled substrates. Later, microautoradiography was combined with cell-specific probing: fluorescent antibodies targeting cell surface antigens of cultured bacteria or fluorescent oligonucleotides targeting sequences of taxonomically revealing ribosomal RNA, often derived from uncultured microorganisms. Recent

efforts (entry 9, Table 6.14) portend another strategy that has the potential of avoiding all laboratory incubations by using microscopic fluorescent in situ hybridization (FISH) procedures to probe naturally occurring microorganisms for both identity (rRNA sequence) and for activity (indirectly, via hybridization with the mRNA of expressed functional genes).

Another promising methodological development is stable isotope probing (SIP; entries 10–16, Table 6.14). The strategy follows the stable isotopic signature of an assimilated substrate (e.g., carbon source) into the populations responsible for substrate metabolism in complex microbial communities. Because the assimilated substrate has a distinctive signature mass (e.g., density or ^{13}C : ^{12}C ratio), cells or biomarkers derived therefrom can be separated and/or analyzed in ways that reveal the identity of active cells (Madsen, 2006, 2011). Without question, the most elegant example of SIP to date is from a series of investigations documenting anaerobic methane oxidation in deep waters adjacent to methane sources in the Black Sea and in coastal California and Oregon (entry 10, Table 6.14; see also Sections 7.4 and 7.5). These investigations were successful because the field study sites contained a substrate (methane) fortuitously labeled with a unique stable isotopic signature. Such situations are rare. To implement SIP in other contexts, a stable isotopically labeled (e.g., ^{13}C) substrate is dosed to a community and later retrieved in biomarkers. Such biomarkers have included phospholipid fatty acids (whose molecular structure is taxonomically informative; see Box 6.2), DNA, and rRNA (the latter two are sources of 16S rRNA gene sequences; see Section 6.7). Early SIP studies established "proof of principle" for the dosing approach – however, these investigations were carried out on enrichment cultures (laboratory-based model soils exposed to high concentrations of ^{13}C-labeled substrates for many weeks). More recently, refinements in the SIP approach have included analyzing the labeled RNA fraction (RNA is rapidly turned over in cells and labeling does not require that the populations undergo growth) and verification of SIP-discovered rRNA sequences with FISH. SIP was applied in a field situation (naphthalene-contaminated sediment), leading to the discovery and later cultivation of an ecologically significant bacterium, *Polaromonas naphthalenivorans* (entry 15, Table 6.14). Field-based SIP has also been used (in conjunction with DGGE (denaturing gradient gel electrophoresis), 16S rRNA gene phylogeny, and phospholipid fatty acid analysis) to show that *Geobacter* species are likely the causative agents of uranium reduction in a uranium bioremediation field project (entry 16, Table 6.14).

Entries 17 and 18 of Table 6.14 elucidate nitrogen transformations in marine habitats. The fundamentals of anaerobic ammonia oxidation (anammox) have been documented only in the last decade (Strous and Jetten, 2004; Kartal et al., 2011; see Sections 7.4 and 7.5). Members of an unusual uncultured group of microorganisms in the *Planctomyces* phylum carry out the anammox reaction. Armed with biomarkers and physiological tools derived from microorganisms in bioreactors that use ammonium as an electron donor and nitrite as an electron acceptor, the anammox

process was found to occur in marine sediments in the Black Sea and off the coasts of Costa Rica and Africa. *Trichodesmium* (entry 18, Table 6.14) is a long-studied photosynthetic and nitrogen-fixing cyanobacterium found in ocean waters. *Trichodesmium* forms relatively large, filamentous, morphologically recognizable colonies whose global presence and potential for N_2 fixation are undeniable. By the strict criteria developed here, N_2 fixation by *Trichodesmium* has not yet been directly demonstrated because the N_2 fixation assay relied upon ship-incubated water samples. Nonetheless, biomarker studies performed on field-fixed samples have shown that nitrogen fixation genes were transcribed and translated in situ.

Entry 19 of Table 6.4 focuses on *Prochlorococcus*, another widely distributed ocean inhabitant that is recognizable by flow cytometry. Representatives of *Procholorococcus* have been cultured and their genomes have been sequenced (e.g., Rocap et al., 2003). In situ photosynthesis by *Prochlorococcus* was demonstrated by field-based monitoring of cell replication.

A two-year field study in oil-contaminated coastal marine mud flats used isolation, physiology, quantitative monitoring of 16S rRNA, and naphthalene dioxygenase mRNA to discover that populations of *Alteromonas* were active in situ in naphthalene degradation (entry 20, Table 6.14). Three metatranscriptomic studies (entries 21–23, Table 6.14) used high-throughput sequencing of community mRNA pools to identify both in situ physiological/biogeochemical processes and the cells catalyzing them. Baker et al. (2013) found that transcripts for nitrite oxidation, amino acid transport, and carbon cycling (and many other processes) were expressed by members of the *Nitrospirae*, *Alteromonas*, and Marine group II *Archaea*. Lesniewski et al. (2012; entry 22, Table 6.14) used metatranscriptomics to link *Nitrosopumilus*/Marine group I *Archaea*, *Methylobacte*r/*Methylomonas*, and the gammaproteobacterium SUP 05, respectively, to in situ oxidation of ammonia, methane, and sulfur in a hydrothermal vent plume. When Stewart et al. (2012) applied metatranscriptomics to waters featuring extreme oxygen depletion (oxygen minimum zone, OMZ) in the Eastern Tropical South Pacific off Peru, these investigators found that key community populations included *Nitrosopumilus*, *Kuenenia*, *Pelagibacter*, and SUP05, while key processes included both aerobic and anaerobic ammonia oxidation, sulfate reduction, sulfur oxidation, and denitrification (entry 23, Table 6.14). The final two entries in Table 6.14 (also described in Section 6.10) provide examples of how metaproteomics has been successfully used to link key populations to key processes. *Bacteria* and *Archaea* in the Southern ocean are responsible for at least seven physiological processes (entry 24, Table 6.14), while five types of fungi in Austrian forest soils are responsible for cellulose degradation (entries 24 and 25 of Table 6.14).

Outlook

The ultimate goals of environmental microbiology are to understand mechanistic relationships between habitat characteristics, evolutionary pressures,

microbial diversity, biochemical processes, and their genetic controls. Processes carried out by microorganisms in soils, sediments, oceans, lakes, and groundwaters have a major impact on environmental quality, agriculture, and global climate change. Thus, environmental microbiological insights have ecological and technological applications able to harness microbial processes that maintain ecosystems, locally and globally.

Identifying ecologically significant microorganisms has traditionally been like finding a needle in an unusual haystack – a haystack whose individual pieces can, during the search, change themselves into misleading needles (Madsen, 2005). For more than a century, environmental microbiologists have been confronted by vast unknown microbial diversity (the "haystack"), by population responsiveness (the misleading "needles"), by an enormous size differential between humans (~1 m) and microorganisms (~1 μm), and by the evasive task of documenting the geochemical impact of microorganisms in open, heterogeneous field sites. *The complexity of natural systems has, almost without exception, made it impossible to directly observe the identity of microorganisms and their activities in waters, sediments, and soils. Instead, indirect approaches have emerged*. These approaches have been described in detail in Figure 6.13 and Table 6.14.

As the frontiers of environmental science and both microbial ecology and evolution advance, we are assured of an astonishing supply of new hypotheses relating microbial diversity to mechanisms of ecologically significant biochemical and physiological adaptation. Current examples include the challenge of discovering the role of uncultured microbes in fresh waters, sediments, oceans, and soils. The new omics and bioinformatics tools are making huge and swift advancements that allow both the microbial players and their ecological (especially biogeochemical) roles to be identified. As these advancements accrue, expect increasingly refined hypotheses to be formulated and tested about mechanistic linkages between ecosystem state variables (temperature, wind, pollution, human management, etc.) and physiolgical processes carried out by naturally occurring microbial populations. The feedback-based investigative strategies available to environmental microbiologists (see Figure 6.13) guarantee complementation, validation, and convergence of information generated by cultivation- and noncultivation-based procedures. Future inquiries will surely accelerate progress linking ecologically important microorganisms to their activities in real-world habitats.

STUDY QUESTIONS

1 Prior to reading this chapter, were you familiar with the term, "epistemology"? Have you noticed that direct observations (e.g., "the sun is up" or "the temperature is 28 °C") are easy to "know" about? In contrast, many types of scientific knowledge cannot be attained through direct observation. Instead, indirect observations, often involving theory and inference, are used to understand many scientific phenomena. Use library publications to research a topic and then write a

two page essay describing some area of science other than environmental microbiology (e.g., biology, physics, chemistry, engineering, medicine) where indirect observations and inference have been successfully and/or routinely used in inquiry. In considering topics for this essay, "think big". Consider issues such as:
(A) Global warming in environmental science.
(B) AIDS, mad cow disease, or prions in medical microbiology.
(C) The Higgs boson in particle physics.

2 Scrutinize Figure 6.1. Enrichment culturing suffers from the potential weakness that, in the end, you have isolated a microorganism that is physiologically interesting but possibly ecologically irrelevant.
(A) Redraw the two bar graphs in Figure 6.1 in a way that shows an outcome of ecological relevance.
(B) If you suspect that you have isolated a bacterium that is ecologically important, how would you prove it?
Formulate your answer as a hypothesis. State the alternative hypothesis or hypotheses. Next state how you would test the hypotheses (see especially Section 6.11).

3 Which one of the quotes in Table 6.1 is most striking to you? Why?

4 Section 6.3 argues that an inability to assemble mass balances hinders our ability to know that microorganisms are responsible for biogeochemical change in a given habitat.
(A) Can you think of a real-world situation where, contrary to the general rule, mass balances *can* be obtained? (Hint: consider the sewage treatment system for a typical small city. Materials enter and leave the treatment plant and this engineered system should allow measurement of concentrations of materials, i.e., total organic carbon or individual pollutant compounds, and their flows – thus allowing mass balances to be assembled.)
(B) Describe a scenario at a sewage treatment plant or another suitable chosen system where monitoring of inputs and outputs can successfully document a microbial process in the plant. What other measurements would strengthen your case for the process?
(C) Next, reconsider the system as a general way to demonstrate microbial metabolic processes. Why might some microbial processes be difficult or impossible to document in your system? (Hint: consider a wide variety of materials that will typically be entering and leaving the system. Physical and chemical properties of the compounds acted upon and produced as metabolites by microbial processes may not be amenable to mass balance strategies.)

5 In Section 6.4, the term "bottle effects" is mentioned.
(A) What do you think "bottle effects" are? In answering, consider the detailed physical and chemical conditions that microorganisms experience at the scale of micrometers.
(B) If you were a free-living, planktonic bacterium in a lake and you were moved into a glass bottle, what physical, chemical, nutritional, and ecological changes might be imposed upon you?

6 (A) In devising a sampling scheme for a microbiological study (see Section 6.5), when would it be absolutely essential to use sterile sampling tools to place the sample into sterile containers?
(B) Can you envision one or more situations in which aseptic sampling techniques would be unnecessary?

7 Does the "capture–fix–store–analyze" scheme in Figure 6.4 make sense to you? If so, why? If not, why not?

8 At the end of the text in Section 6.5, a methane-rich site is mentioned. How would you distinguish between microbiologically created methane and methane of geologic origin (from natural gas)? To answer this question, consider information provided in all prior

chapters – especially Chapter 2 (Section 2.1 and Box 2.2) and Section 6.8. Include arguments using both analytical chemistry procedures and physiological considerations of the habitat.

9 Section 6.7 advances the idea that a microorganism in low abundance might be physiologically and ecologically important.
   (A) Can you explain this idea?
   (B) Do active microorganisms necessarily have high growth yields? In preparing an answer, consider information provided in Chapter 3 – especially the thermodynamic "compass" used to identify processes that may have low free-energy yields.
   (C) Find one or more electron donor–electron acceptor reaction pairs (e.g., Figure 3.10 and Tables 3.7 and 3.8) that you consider to be good candidates for processes carried out by microorganisms in ecosystems that meet both of the following criteria: (i) small microbial populations and (ii) large fluxes of substrate turnover. What are the processes you have chosen?

10 In Figure 6.7, one experimental scheme converts rRNA (initially present as intracellular ribosomes) to cDNA prior to cloning and sequencing. Explain why investigators would choose this reverse transcriptase-based step rather than an experimental approach that uses rRNA genes (DNA) that encode the rRNA for PCR amplification, cloning, and sequencing.

11 Section 6.7 mentions the possibility of finding rRNA genes in reagent-only treatments. In this regard, "control" treatments in experimental designs are crucial. It would be tragic to mistake microorganisms in the PCR and cloning reagents for those in the habitat you are sampling. If you were analyzing rRNA genes from microorganisms collected from a low-biomass habitat, such as air in a hospital, what experimental controls would help you have confidence in the data you produce? To answer this question, assume that 100 l volumes of hospital air are sampled using 0.1 μm pore-size membrane filters housed in a canister.

12 Boxes 6.2 and 6.3 describe lipid biomarkers and 16S rRNA fingerprinting procedures, respectively. Information in Figure 6.5 places these biomarker procedures within the larger context of the environmental microbiology "toolbox". If you wanted to answer the question "What microorganisms live on my hand?" what methods would you use? Justify your choices. Presume that you have access to all instruments and assays described in this chapter and the rest of this book.

13 Having completed the exercise in question 12, assume that you now have a description of the microbial community that dwells on your hand. Now you are confronted with the task of discovering "What are they doing?" and "When are they doing it?" Devise a series of experimental procedures for answering these two questions. Begin with clear hypotheses, then decide how the hypotheses should be tested, and then describe the experimental steps that would need to be implemented. Take these steps through to completion – beginning with sampling and ending with final analysis of the data. Be careful to consider alternative hypotheses for interpreting each set of measurements and to take steps that avoid potential artifacts in your procedures. In devising this experimental plan, use Figure 6.5 as an overall guide and feel free to utilize analytical, molecular, cultivation, metagenomic, and omic procedures.

14 Section 6.10 provides a broad overview of the types of information that "next-generation omics technologies" can generate for environmental microbiologists.
   (A) Prepare a list of 2 or 3 advantages over "traditional sequencing" procedures for each technology (barcode-based small-subunit rRNA, metagenomics, metatranscriptomics, and metaproteomics).
   (B) Can you think of any disadvantages of these technologies?
   (C) If you had the task of understanding the microbial ecology of a field study site (such as Yellowstone Hot Springs or ocean sediments) and access to only one of the next-generation omics procedures described in Section 6.10, which one would you use? Explain your reasoning.

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7

Microbial Biogeochemistry: A Grand Synthesis

- *What is the "stuff of life"?*
- *What materials are passed among inhabitants of the biosphere as they are born, grow, replicate, and die?*
- *What developments have occurred over evolution that incorporate elements of rocks and minerals into biological systems?*
- *How dynamic are the pools of nutrients? How are they consumed and how are they regenerated?*
- *What drives vital processes of metabolism, heredity, and evolution?*

Biogeochemistry helps to answer these and other questions related to biosphere function and maintenance. In earlier chapters of this text, broad issues of biogeochemistry were introduced. These issues have included the following.

Chapter 1
- *Earth habitats as heterogeneous gradients of environmental conditions.*
- *An overview of nutrient cycling and microbial physiological processes (see Tables 1.2 and 1.4).*
- *The complexity of dynamic real-world habitats and the notion of budgets and models to help understand them (see Figures 1.3 and 1.4).*

Chapter 2
- *Events that occurred during the Earth's geochemical development over 4.6 × 10^9 years (see Table 2.1).*
- *The role of hydrophobic metal-sulfide mineral surfaces both in catalyzing energy-capturing reactions and in assembling membrane-bound compartments crucial for early life (see Sections 2.4 to 2.6 and Figures 2.3 to 2.6).*
- *The rise of oxygen in the biosphere, including the geologic evidence and biological and evolutionary consequences (see Sections 2.8 to 2.11, Table 2.1, and Figures 2.1, 2.2, and 2.7 to 2.12).*

Chapter 3
- *The role of carbon and energy sources in determining five types of microbial nutrition (see Tables 3.3 and 3.4).*

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- *Ecosystem nutrient fluxes that regulate the physiological status and composition of microbial communities (see Table 3.4 and Figure 3.6).*
- *The biosphere as complex mixtures in thermodynamic disequilibrium (see Figures 3.8 and 3.9).*
- *The thermodynamic hierarchy of half reactions that makes sense of the biosphere's many possible reaction pathways (see Tables 3.7, 3.8, Figures 3.9, 3.10, and Boxes 3.3 to 3.8).*

Chapter 4

- *A survey of the biosphere's biomes, soil types, and aquatic and subsurface characteristics (see Tables 4.1 to 4.3 and Figures 4.1 to 4.11).*
- *A description of the geochemistry and microscale topography of the soil habitat (Figures 4.12 to 4.14).*

Against the backdrop of the above issues from Chapters 1 to 4, we surveyed the diversity of microbial life (Chapter 5) and the methods used to ask key questions in environmental microbiology (Chapter 6).

Here in Chapter 7, we assess six unifying themes in microbial biogeochemistry: (i) the critical role of minerals (especially metals) in the key life processes of enzymatic reactions and toxicity; (ii) the foundations of global climate change; (iii) the major biosphere pools of nutrients and their turnover; (iv) details of several biogeochemical cycles (carbon, sulfur, nitrogen); (v) how and why to explore fundamental biochemical mechanisms of microbial biogeochemical processes; and (vi) global mass balances for carbon, sulfur, and nitrogen.

Chapter 7 Outline

7.1 Mineral connections: the roles of inorganic elements in life processes
7.2 Greenhouse gases and lessons from biogeochemical modeling
7.3 The "stuff of life": identifying the pools of biosphere materials whose microbiological transformations drive the biogeochemical cycles
7.4 Elemental biogeochemical cycles: concepts and physiological processes
7.5 Cellular mechanisms of microbial biogeochemical pathways
7.6 Mass balance approaches to elemental cycles

7.1 MINERAL CONNECTIONS: THE ROLES OF INORGANIC ELEMENTS IN LIFE PROCESSES

Chapter 2 (Sections 2.5 and 2.6) highlighted current theory that metal-sulfide surfaces within porous hydrothermal vent deposits (or geothermal freshwater ponds; see Section 2.6) were likely the site of early biosynthetic reactions and assembly of organic molecules. In contemporary prokaryotic (and eukaryotic) enzymes, iron-sulfur clusters (Figure 7.1) may be the biochemical remnants of ancient physiology. Manifest as reaction centers, iron-sulfur clusters are extremely important in a large variety of proteins involved in oxidation/reduction reactions (essential to electron transport, respiration, and adenosine triphosphate (ATP) generation), as well as proteins that add water and oxygen (as hydratase and oxygenase enzymes, respectively) to organic molecules (Wackett et al., 1989).

One way to develop an appreciation for the importance of inorganic elements (minerals) in microbial processes is based simply on bulk cellular composition. Elemental analysis of dehydrated microbial cells shows that carbon, oxygen, nitrogen, hydrogen, and phosphorus comprise 95% of cell

mass (Wackett et al., 1989). Incorporated into the integrated organic matrix of a cell, the supply of inorganic nutrients can control (limit) the amount of biomass produced during microbial growth. In this way, nutritionally essential elements are defined (Table 7.1). Table 7.2 provides a summary of the elemental components of microbial cells and examples of their physiological roles. While the macronutrients (C, H, O, N, P, S, K, Mg, Ca, Na) play rather obvious roles as components of major subcellular structures or in osmotic balance, it is the micronutrients that reveal, in striking ways, how the unique reactivities of mineral matter (especially metals) contribute to vital life processes.

Metals are required as cofactors in approximately two-thirds of all enzymes (Devlin, 2001). This fact is nothing short of remarkable. Please take a moment to ponder and appreciate this as it is restated: catalysts that drive two-thirds of your own metabolic processes (from nerve transmission to respiratory ATP production to muscle contractions) and two-thirds of the microbially mediated processes that maintain the biosphere *require* the types of metallic micronutrients listed in the lower portion of Table 7.2, including ecologically crucial biogeochemical processes in the oceans (Morel and Price, 2003). *Without inclusion of the metals within the enzymatic structures, the catalysts would be ineffective*. Thus, there are extremely strong links between inorganic components of the geosphere and the evolution of life (from prebiotic Earth to the iron-sulfur world to the RNA and DNA worlds; see Chapter 2) through to contemporary biosphere function.

- **What properties of metals make them so useful for enzymatic catalysis?**

The answer is found in their fundamental atomic characteristics – including atomic mass, ionic radius, charge, oxidation/reduction properties, and the configurations of electrons. Each metal atom possesses a unique combination of electrons in its atomic orbitals. These establish the element's oxidation/reduction properties and

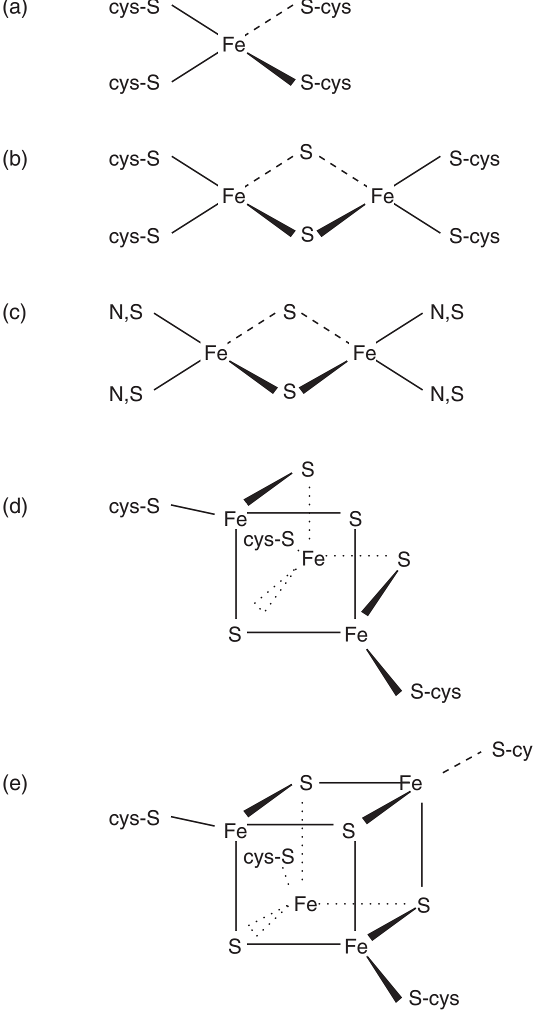


Figure 7.1 Iron-sulfur clusters serve as crucial reactive sites in the enzymes of contemporary organisms. Shown are: (a) a one-iron cluster as found in the rubredoxin protein from *Pseudomonas oleovorans*; (b) a spinach-type 2Fe2S cluster containing four cysteine-sulfur bridging groups (ligands); (c) a Rieske-type 2Fe2S cluster that contains two nitrogen-containing amino acid bridging groups; (d) a 3Fe4S cluster; and (e) a 4Fe4S cluster. (From Wackett, L.P., W.H. Orme-Johnson, and C.T. Walsh. 1989. Transition metal enzymes in bacterial metabolism. In: T.J. Beveridge and R.J. Doyle (eds), *Metal Ions and Bacteria*, pp. 165–246. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York.)

Table 7.1
Bacterial mineral nutrition and growth: approximate amount of a given element (g) to give 100 g of dry biomass. (From Hughes, M.N. and R.K. Poole. 1989. *Metals and Micro-organisms*, table 1.1, p. 3. Chapman and Hall, London. With kind permission of Springer Science and Business Media)

Element	g/100 g
K	1.7
Mg	0.1–0.4
Ca	0.1
Mn	0.005
Fe	0.015
Co	0.001
Cu	0.001
Zn	0.005
Mo	0.001

its three-dimensional configuration for bonding (e.g., octahedral, tetrahedral, square pyramidal) with functional groups in enzymes (amino acid moieties, other ligands). In turn, each particular combination of organic–metal complex in a given enzyme influences the specificity of substrate binding and the reactions catalyzed (Hughes and Poole, 1989). There is a broad selection of inorganic elements (their atomic numbers increase monotonically across the periodic table). There is an even broader, widely varying array of biochemically synthesized organic structures available to form metalloenzymes and other metal–organic molecular complexes within cells. Ultimately, the catalytic and other properties of metal–organic complexes reflect the many available combinations of both inorganic elements and their associated organic molecules.

Wackett et al. (2004) have recently provided a comprehensive overview of the role of all chemical elements in biological, especially microbiological, processes (Figure 7.2). The color-coded patterns in Figure 7.2 expand upon the information provided in Table 7.2 by displaying elements that are "transported, reduced, and/or methylated in some microbes", in addition to those that serve as macro- and micronutrients. When all the biologically active elements are tallied (all but the white boxes in Figure 7.2), the total is 57. Please note that the periodic table in Figure 7.2 omits elements with atomic numbers above 86 (e.g., francium 87) and both the lanthanide and actinide series.

The presence in microorganisms of element-specific, genetically encoded transport mechanisms further confirms the biological and evolutionary significance of the targeted elements. Well-characterized nutrient-uptake

Table 7.2

Overview of the approximate elemental composition of microbial cells and the physiological function of each element. (From Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, table 5.2, p. 105. Prentice Hall, Upper Saddle River, NJ. Reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ. With contributions from Stanier et al., 1986; Hughes and Pool, 1989; Neidhardt et al., 1990; Wackett et al., 2004; Schaechter et al., 2006)

Element	% dry weight	Examples of cellular function
Macronutrients		
Carbon (C)	50	Building blocks of all macromolecules, carbohydrates, organic acids, proteins, lipids, cell walls, cell membranes, etc.
Hydrogen (H)	8	
Oxygen (O)	20	
Nitrogen (N)	14	Proteins, nucleic acids
Phosphorus (P)	3	Nucleic acids, phospholipids, ATP
Sulfur (S)	1	Amino acids (cysteine, methionine), vitamins, coenzyme A
Potassium (K)	1	Osmotic control, enzyme cofactor, ion balance
Magnesium (Mg)	0.5	Stabilization of macromolecular structure (ribosomes, membranes, nucleic acids), enzyme cofactor
Calcium (Ca)	0.75	Cell wall stability, enzyme cofactor
Sodium (Na)	1	Osmotic control, nutrient transport
Micronutrients*		
Iron (Fe)	0.2	Cytochromes, catalases, peroxidases, iron-sulfur proteins, oxygenases, all nitrogenases
Boron (B)	<0.01	Present in autoinducers for quorum sensing in bacteria; also found in some polyketide antibiotics
Chromium (Cr)	<0.01	Required by mammals for glucose metabolism; no known microbial requirement
Cobalt (Co)	<0.01	Vitamin B_{12} transcarboxylase (propionic acid bacteria)
Copper (Cu)	<0.01	Respiration, cytochrome *c* oxidase; photosynthesis, plastocyanin, some superoxide dismutases
Manganese (Mn)	<0.01	Activator of many enzymes; present in certain superoxide dismutases and in the water-splitting enzyme in oxygenic phototrophs (photosystem II)
Molybdenum (Mo)	<0.01	Certain flavin-containing enzymes, some nitrogenases, nitrate reductases, sulfite oxidases, DMSO-TMAO reductases, some formate dehydrogenases
Nickel (Ni)	<0.01	Most hydrogenases, coenzyme F_{430} of methanogens, carbon monoxide dehydrogenases, urease
Selenium (Se)	<0.01	Formate dehydrogenase, some hydrogenases, amino acid selenocysteine
Tungsten (W)	<0.01	Some formate dehydrogenases, oxotransferases of hyperthermophiles
Vanadium (V)	<0.01	Vanadium nitrogenase, bromoperoxidase
Zinc (Zn)	<0.01	Carbonic anhydrase, alcohol dehydrogenase, RNA and DNA polymerases, many DNA-binding proteins

DMSO, dimethylsulfoxide; TMAO, trimethylamine oxide.

*Not every micronutrient listed is required by all cells; some metals listed are found in enzymes present in only specific microorganisms.

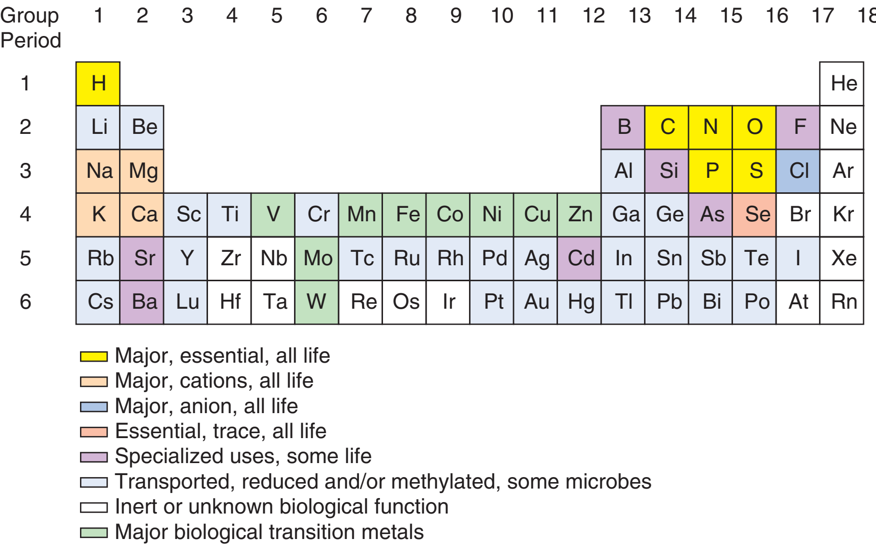


Figure 7.2 Classification of the elements of the periodic table based on their role in biological, especially microbiological, processes. (From Wackett, L.P., A.G. Dodge, and L.B.M. Ellis. 2004. Microbial genomics and periodic table. *Appl. Environ. Microbiol.* **70**:647–655. With permission from the American Society for Microbiology.)

transport systems in prokaryotes include those for K^+, Mg^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Na^+, PO_4^{3-}, and SO_4^{2+} (Silver, 1998). In contrast, a broad variety of naturally occurring elements can be toxic to microbial (and other) cells. These pose selective pressures of another kind: microbial adaptations eliminate toxicity by transforming the element or by mobilizing the element away from the cell. The detoxification mechanisms are manifest as efflux pumps, reduction, and/or methylation (Silver, 1998). A group of elements drawn from the light blue and green areas of Figure 7.2 that are detoxified by well-characterized specific, genetically encoded processes include: Hg, As, Sb, Cd, Zn, Co, Ni, Ag, Cu, Cr, Fe, and Pb (Table 7.3).

7.2 GREENHOUSE GASES AND LESSONS FROM BIOGEOCHEMICAL MODELING

One of the most important twentieth century developments in global biogeochemistry has been alterations in the chemical composition of Earth's atmosphere: a buildup of tropospheric (low-altitude) greenhouse gases. Microorganisms significantly influence this issue. Reviewing the greenhouse effect, global warming, and accompanying climate change provides a means to illustrate critical principles that apply to all biogeochemical concerns.

Table 7.3

Nine classes of inorganic compounds that are toxic to microorganisms and their corresponding genetic and physiological detoxification mechanisms. (From Silver, S. 1998. Genes for all metals – a bacterial view of the periodic table. *J. Indust. Microbiol. Biotechnol*. **20**:1–12, fig. 2. With kind permission of Springer Science and Business Media)

Class	Element/compound	Mechanism of detoxification or resistance*
1	Hg^{2+}	*mer*. Hg^{2+} and organomercurials are enzymatically detoxified via cleavage redox reactions that mobilize the compounds away from the cell (see Table 7.4 in Section 7.3)
2	AsO_4^{3-}, AsO_2^{-}, SbO^{+}	*ars*. Arsenate is enzymatically reduced to arsenite by ArsC. Arsenate and antimony are "pumped" out by the membrane protein ArsB that functions chemiosmotically alone or with the additional ArsA protein as an ATPase
3	Cd^{2+}	*cadA*. Cd^{2+} (and Zn^{2+}) are pumped from Gram-positive bacteria by a P-type ATPase with a phosphoaspartate intermediate
4	Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}	*czc*. Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} are pumped from Gram-negative bacteria by a three-polypeptide membrane complex that functions as a divalent cation/$2H^{+}$ antiporter. The complex consists of an inner membrane protein (CzcA), an outer membrane protein (CzcC), and a protein associated with both membranes (CzcB)
5	Ag^{+}	*sil*. Ag^{+} resistance results from pumping from bacteria by three-polypeptide chemiosmotic exchangers plus a P-type ATPase
6	Cu^{2+}	*cop*. Plasmid Cu^{2+} resistance results from a four-polypeptide complex, consisting of an inner membrane protein, an outer membrane protein, and two periplasmic copper-binding proteins. In *Pseudomonas*, cop results in periplasmic sequestration of Cu^{2+}. In addition, chromosomally encoded P-type ATPases provide partial resistance by effluxing Cu^{2+} or Cu^{+}
7	CrO_4^{2-}	*chr*. Chromate resistance results from a single membrane polypeptide that causes reduced net cellular uptake, but efflux has not been demonstrated
8	TeO_3^{2-}	*tel*. Tellurite resistance results from any of several genetically unrelated plasmid systems. Although reduction to metallic Te^{0} frequently occurs, this does not seem to be the primary resistance mechanism
9	Pb^{2+}	*pum*. Lead resistance appears to be due to an efflux ATPase in Gram-negative bacteria and the accumulation of intracellular $Pb_3(PO_4)_2$ in Gram-positive bacteria

*The involved genetic system is italicized (e.g., *mer* are mercury-resistance genes and *ars* are arsenic-resistance genes). Enzymes encoded by particular genes are shown in nonitalicized letters: for example ArsC is the structural enzyme, arsenate reductase, encoded by the *arsC* gene. Resistance systems await understanding for bismuth (Bi), boron (B), thallium (TI), and tin (Sn).

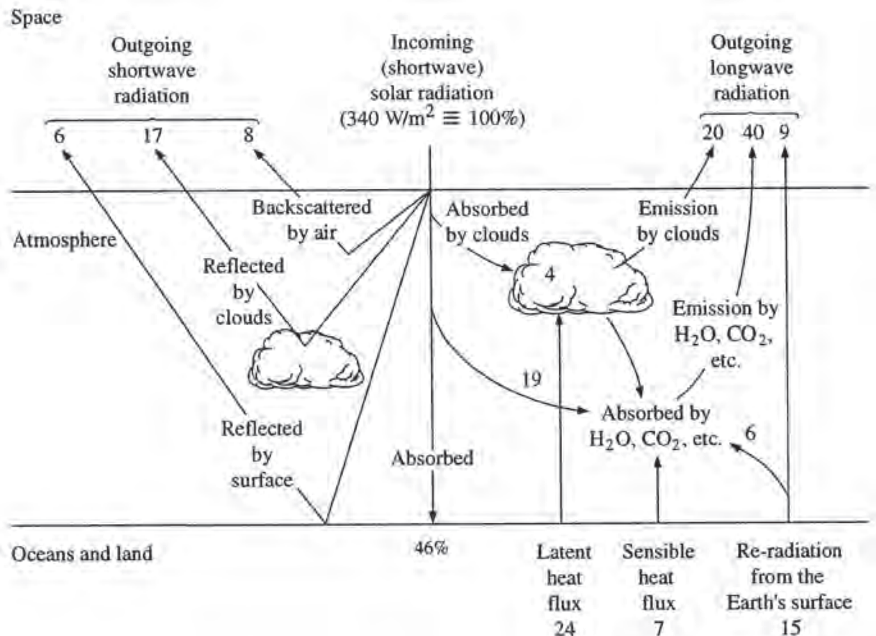


Figure 7.3 The radiation budget for Earth. Latent heat is heat stored in water as it changes from liquid to the vapor phase. Sensible heat is convection, especially from the oceans to the atmosphere. See text for details. (Reprinted from Schlesinger, W.H. 1997. *Biogeochemistry: An Analysis of Global Change*, 2nd edn. Academic Press, New York. Copyright 1997, with permission from Elsevier.)

Problem: global warming

The Earth's lower atmosphere, the troposphere (up to ~15 km thick; Schlesinger, 1997), consists of a blanket-like layer of gases that keeps the Earth warm. Figure 7.3 provides an overview of the radiation budget for our planet. Incoming light delivers energy at a rate (or power) of 340 W/m^2 to the outer atmosphere. A portion of the incident light (~30%) is reflected back into outer space, largely unaltered. The remainder interacts with global materials (gases, clouds, water, land, biota), being entirely absorbed or absorbed and then released back to space as outgoing light of longer wavelength (~70% of the incident power). Without the naturally occurring greenhouse gases to retain heat, Earth's temperature would drop by 33°C (Henshaw et al., 2000), causing substantial conversion of liquid water to ice.

As shown in Figure 7.3, the major greenhouse gases contributing to storage of heat in the troposphere are H_2O (approximately two-thirds of storage) and CO_2 (about one-third). However, during the last century, several naturally occurring greenhouse gases (e.g., CO_2, methane, nitrous oxide, ozone) as well as a variety of synthetic compounds known as

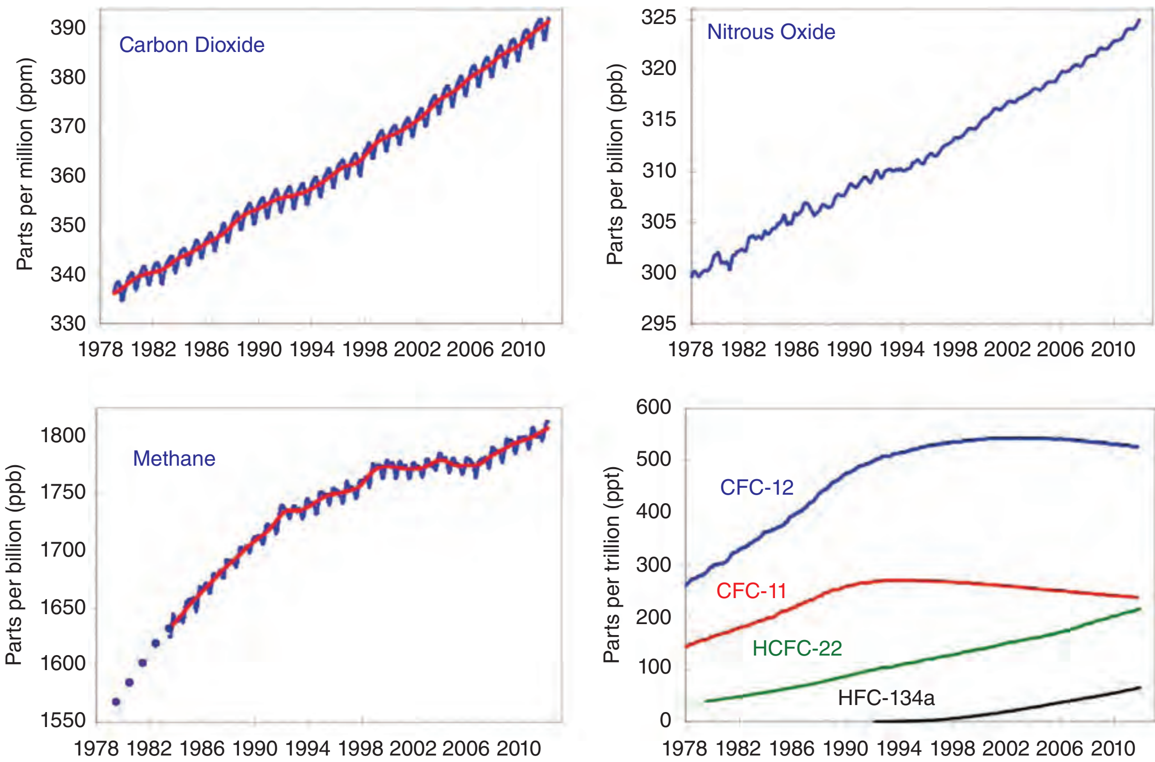


Figure 7.4 Historical records of the concentrations of seven atmospheric gases important for global warming that occur in the troposphere. CFC-12 (CCl_2F_2) and CFC-11 (CCl_3F) also contribute to stratospheric ozone destruction. Data are for the northern hemisphere. Oscillations (CO_2, methane) reflect how the balance between destruction and production fluctuate annually. See text for details. (From D. Hofmann, NOAA, with permission.)

chlorofluorocarbons (CFCs) have increased significantly above historical levels (Figure 7.4; Houghton et al., 2001).

The overall rise in tropospheric carbon dioxide (~18%) and methane (~16%) during the last 35 years (Figure 7.4) display annual sinusoidal oscillations reflecting a seasonal shift between processes that produce and consume each of the gases. In the case of CO_2, the two opposing processes are clearly photosynthesis and respiration, the former causing an annual decline in CO_2 concentration in the northern hemisphere each summer (Schlesinger, 1997). The long-term rising trend in CO_2 concentration is the result of fossil-fuel burning (largely coal and petroleum products) – which releases CO_2 at a rate that exceeds the rate of return to nonatmospheric global carbon reservoirs. In the case of methane (Figure 7.4), its 2.5-fold rise from its pre-industrial atmospheric concentration (of 700 ppb) to contemporary (~1794 ppb) is well documented (Dlugokencky et al., 2011). A variety of human-managed systems (e.g., wetland rice production, burning of vegetation, herds of ruminant livestock (whose anaerobic digestive

tracts harbor methanogenic bacteria), industry, processing of fossil fuels, and mining operations) are almost certainly driving the global increase in tropospheric methane (Dlugokencky et al., 2011). A major potential mechanism of methane consumption is microbial metabolism: both aerobic and anaerobic microorganisms in soils, waters, and sediments use methane as a source of energy (ATP production) and/or carbon (see Sections 3.11, 7.4, 7.5, and Box 7.4). Because these microbial processes are subject to annual activation by warm temperatures, they likely contribute to the annual oscillations shown in Figure 7.4. The other major (and nonseasonal) loss mechanism for tropospheric methane involves scavenging in the atmosphere by gas-phase hydroxyl radicals (Schlesinger, 1997).

The perceptive reader will note that the vertical scale units for CO_2 concentration is parts per million (ppm) in Figure 7.4, while the scale for the other four compounds is smaller by a factor of 10^3 (parts per billion) or 10^6 (parts per trillion).

- **Why would relatively small rises in tropospheric concentrations of nitrous oxide (N_2O) and synthetic gases used by the refrigeration industry (CFC-12, CFC-11, HCFC-22, HFC-134a, CCl_2F_2, CCl_3F, HCF_2Cl, and CH_2FCF_3), respectively, be of global significance?**

The reason the greenhouse effect responds to trace levels of CH_4, N_2O, CFCs, HCFCs, and HFCs lies in two key details of their infrared absorption spectra (the main mechanism of atmospheric heat retention; Charlson, 2000):

1. These gases (Box 7.1) absorb strongly within that part of the infrared spectrum where water vapor and CO_2 do not already absorb.
2. This portion of the spectrum is also a major avenue of Earth's loss of radiant energy.

The seven gases shown in Figure 7.4 (plus trophospheric ozone, see below) are estimated to account for nearly all of the global warming that has occurred since industrialization began in ~1750 (Charlson, 2000; Houghton et al., 2001). Information in Figure 7.5 (from the Intergovernmental Panel on Climate Change (IPCC) organized by the World Meteorological Organization and the United Nations Environmental Programme) provides an integrated glimpse of the diversity of forces that can alter the Earth's radiation balance (radiative forcing in Figure 7.5 has units of W/m^2 of the globe's surface areas). Greenhouse gases and tropospheric ozone constitute only two of nearly a dozen other factors that may cool or warm the atmosphere. Among the others are aerosols, aviation-induced clouds (contrails), land use, and variability in incident solar energy. As is shown in the right-hand column of Figure 7.5, the level of scientific understanding (LOSU) is higher for the influence of greenhouse gases than any of the other factors.

Box 7.1

Greenhouse gases: on a molar basis their effects are not equal (from Rodhe, H. 1990. A comparison of the contribution of various gases to the greenhouse effect. *Science* 248:1217–1219. Reprinted with permission from AAAS; updated)

Type of gas	Contribution, relative to CO_2, of various greenhouse gases to the greenhouse effect
CO_2	1
CH_4	25
N_2O	200
Ozone (O_3)	2,000
CFC-11 (CCl_3F)	12,000
CFC-12 (CCl_2F_2)	15,000
HCFC-22 (HCF_2Cl)	1800
HFC–134a (CH_2FCF_2)	1300

The impact of each particular greenhouse gas on global warming results from a combination of:

- The compound's infrared absorption spectrum.
- The amount of energy at a given wavelength already absorbed by water and CO_2.
- The intensity of potential radiation loss from Earth to outer space at a given wavelength.

Note: –Chlorofluorocarbon (CFCs) have been banned due to their Cl content, and hence their ability to destroy stratospheric ozone.
–Hydrochlorofluorocarbons were introduced because of their lower Cl content.
–Hydrofluorocarbons (HFCs) do not destroy stratospheric ozone.

(See text for details.)

Implicit in coping with greenhouse gas-induced climate change is recognizing that true change has occurred. Figure 7.6 (from the 2007 IPCC report) provides a 10,000-year record of atmospheric concentrations of carbon dioxide, methane, and nutrient oxides. This puts contemporary, post-industrialized planetary conditions into a historical context. It is undeniable that all three of these very powerful greenhouse gases have become dramatically enriched in the Earth's atmosphere since industrialization began (~1800, see inserts in the three panels of Figure 7.6).

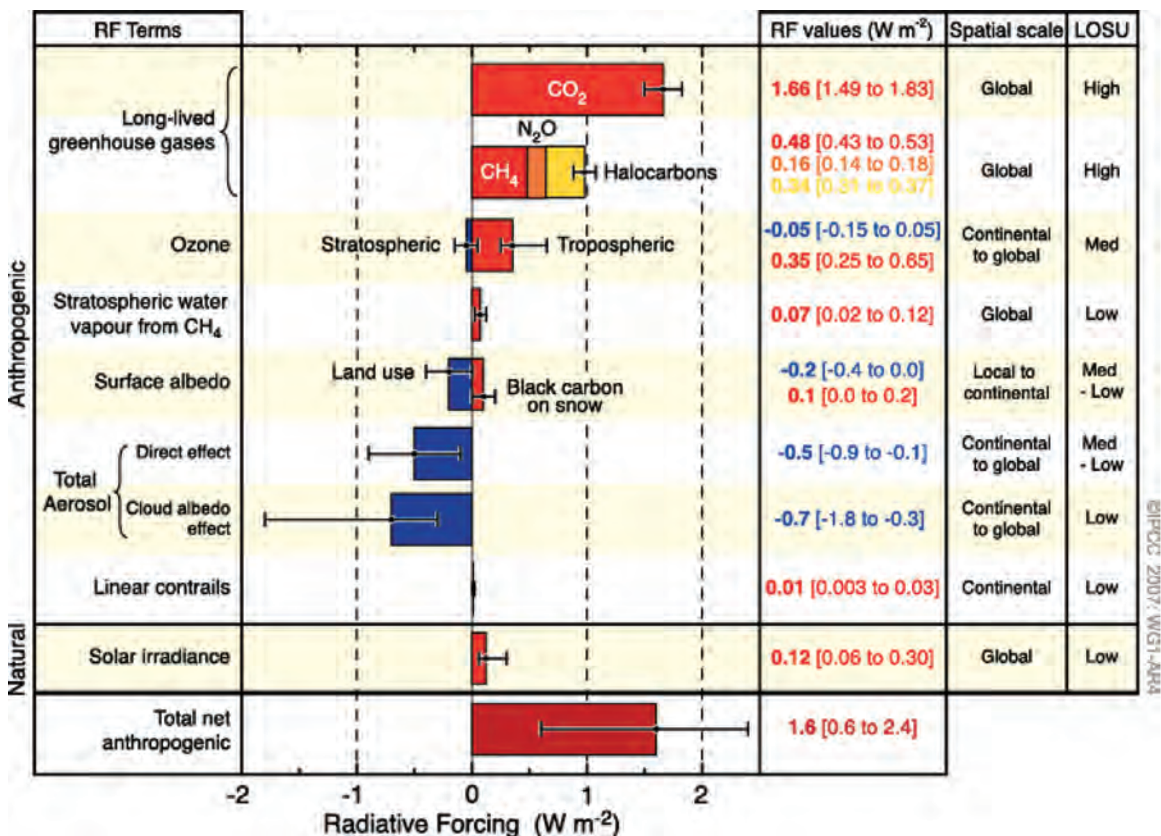


Figure 7.5 Seven interacting factors (including long-lived greenhouse gases, ozone and aerosols) that govern the radiation balance in the Earth's atmosphere. Global average radiative forcing (RF) estimates and ranges in 2005 for each factor, together with the typical geographical extent (spatial scale) of the forcing and the associated level of scientific understanding (LOSU) for each is shown along the horizontal axis. (From Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor, and H.L. Miller. 2007. *Contribution of Working Group I to the Fourth Assessment of the International Panel on Climate Change, 2007*. Cambridge University Press, Cambridge, UK. With permission from the Intergovernmental Panel on Climate Change.)

One major issue confronting scientists, environmental managers, planners, and policy-makers is distinguishing "true climate change" from "natural variability in climate conditions" – especially at the local level. Early public, institutional and governmental recognition of climate change is critical – as stabilization of Earth's bioclimate system can only be achieved via cooperation among people across the globe. Addressing the issue of "perceived" versus "real" climate change, Hansen et al. (2012) have completed a rigorous statistical analysis of high-temperature localized hot spells (anomalies of extreme heat) using very recent history (1951 to 1980) as the reference baseline. Hansen et al. (2012) concluded that several extreme heat anomalies in 2010 and 2011 (from Texas to Moscow) were a

consequence of global warming because their likelihood in the absence of global warming was exceedingly small. Human-induced global climate change is one of the most pressing issues facing humanity (Moss et al., 2010; Solomon et al., 2007).

Lessons from modeling biogeochemical cycles

A variety of naturally occurring processes at the interface between the geosphere, atmosphere, and hydrosphere are cyclic in nature:

- Water evaporates and then condenses as precipitation.
- Continents erode into the oceans and sediments are then uplifted by crustal movements.
- Photosynthesis removes carbon dioxide from the atmosphere and then respiration returns it.

Models are tools that allow cyclic processes to be formally recognized. Then information about particular phenomena can be systematically assembled and used to: (i) interpret historical events and (ii) predict future events (Rodhe, 2000). By formally declaring facts about and relationships between components of biogeochemical systems, models (especially quantitative models) are heuristic – they

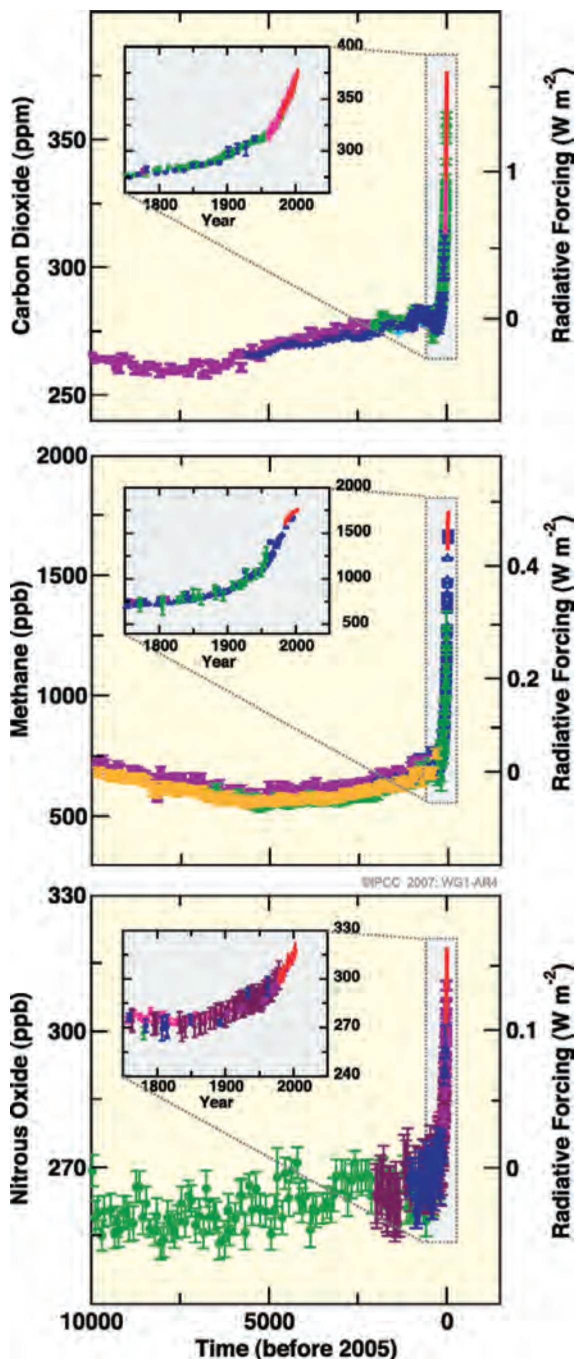


Figure 7.6 Atmospheric concentrations of carbon dioxide, methane and nitrous oxide over the last 10,000 years (large panels) and since 1750 (inset panels). Measurements are shown from ice cores (symbols with different colours for different studies) and atmospheric samples (red lines). The corresponding radiative forcings are shown on the right-hand axes of the large panels. (From Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor, and H.L. Miller. 2007. *Contribution of Working Group I to the Fourth Assessment of the International Panel on Climate Change, 2007*. Cambridge University Press, Cambridge, UK. With permission from the Intergovernmental Panel on Climate Change.)

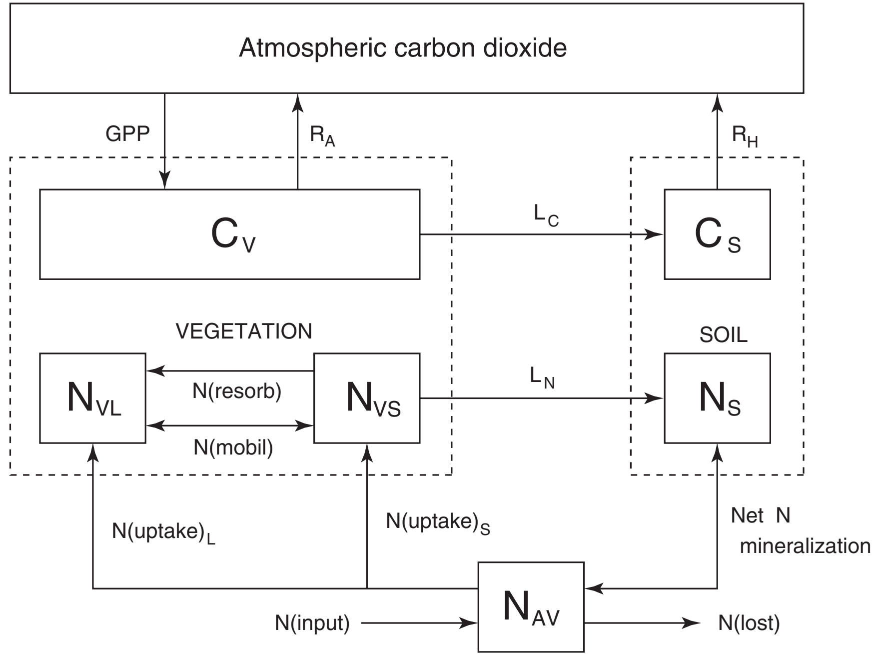


Figure 7.7 Conceptual biogeochemical model of carbon and nitrogen flow in global terrestrial habitats. This terrestrial ecosystem model consists of boxes representing three pools of carbon and four pools of nitrogen dispersed across three environmental compartments: the atmosphere, vegetation, and soil. C_S, C_V, carbon in soil or vegetation; L_C, L_N, litter carbon or nitrogen; GPP, gross primary productivity; N_S, N_V, nitrogen in soil or vegetation; N_{VL}, labile nitrogen in vegetation; N_{VS}, structural nitrogen in vegetation; N_{AV}, available inorganic nitrogen in soil; R_A, R_H, respiration by autotrophs or heterotrophs. (From Xiao, S., D.W. Kicklighter, J.M. Melillo, A.D. McGuire, P.H. Stone, and A.P. Sokolov. 1997. Linking a global terrestrial biogeochemical model and a 2-dimensional climate model: implications for the global carbon budget. *Tellus* B **49**:18–37. With permission from Blackwell Publishing, Oxford, UK.)

reveal knowledge gaps and inaccuracies, and identify new areas of inquiry. In turn, the newly created information can be funneled into improved models that lead to an increased understanding of the system of interest.

On paper, conceptual biogeochemical models (e.g., of a lake or a rainforest or the entire globe) resemble a series of boxes connected by arrows. An example is shown in Figure 7.7 – the visual depiction of carbon and nitrogen compounds as they function in terrestrial habitats. A variety of ecosystem processes cause carbonaceous and nitrogenous substances to be passed between pools of these compounds in soil and plants (vegetation). In Figure 7.7, carbon mineralization (conversion to CO_2) is shown, as is nitrogen

Box 7.2

Issues common to models of all biogeochemical processes (compiled from Rodhe, 2000; see also Wang et al., 2007, 2010, and Zaehle and Dalmonech, 2011)

- Reservoirs (pools of materials) = M.
- Fluxes = mass transferred between reservoirs per unit time.
- Source (flux into reservoir) = Q.
- Sink (flux out of reservoir) = S.
- Budget = balance sheet of sources, sinks.
- Reservoir turnover time $T = M/S = M/Q$ in steady state.
- Residence time (of a molecule in a given pool of material) = probability-based calculation of how long a molecule of substance will take to exit the system once it has entered.
- Response time (of a reservoir) = timescale that characterizes the adjustment to equilibrium after a sudden change in the system.
- Linearity of system response (to perturbations): responses may be *linear* (directly proportional to input rates or to the degree of change) or responses may be *nonlinear* (by departing from linear responses in unpredictable ways).
- Is the system in steady state?
- What are the system transport and mixing characteristics?
- The system must be well defined, including: boundaries, timescales, and spatial scales.

mineralization to inorganic forms such as ammonia and nitrate (the available nitrogen pool, N_{AV}). One of the key challenges for biogeochemical modeling is to quantify the types and sizes of nutrient pools and the fluxes (arrows in Figure 7.7) between them. Information in Box 7.2 elaborates on some of the terms and concepts pertinent to developing biogeochemical models.

Xiao et al. (1997) have woven the terrestrial ecosystem model shown in Figure 7.7 (itself, a highly complex computer program) into a tapestry of other computer programs aimed at describing the global response of plant communities to climate change across 18 different biomes. The terrestrial ecosystem model works on a spatial grid of >62,000 cells that map the Earth's surface (each ~55 × 55 km in size). In the computer program, calculations for this set of cells are fed into a series of additional atmospheric chemistry and climate models that are coupled together (Figure 7.8). This mega model is designed to integrate the detailed interactions between vegetation, soil, and greenhouse gases – thereby predicting how an anticipated rise in tropospheric CO_2 concentration will influence the global carbon balance – especially the distribution of carbon in pools of plant biomass and soil detritus (soil organic carbon and humus). Additional information about more recent global biogeochemical modeling approaches are described by Wang et al.

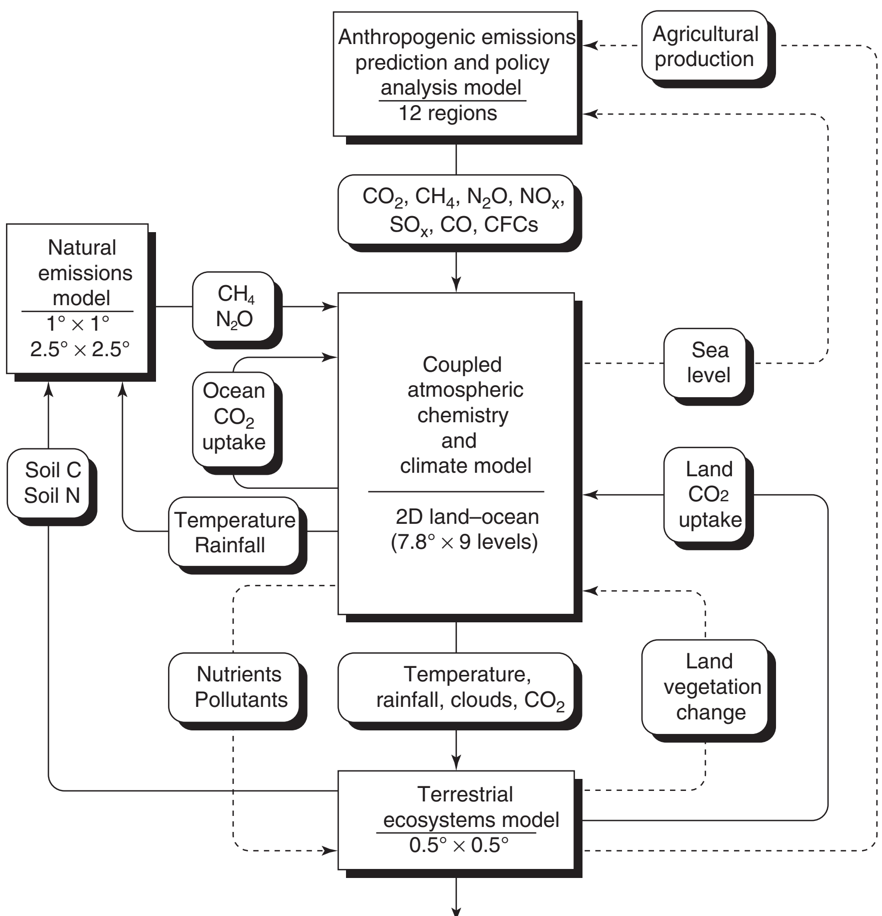


Figure 7.8 Global climate change mega model created by the integrated assessment framework at Massachusetts Institute of Technology. The goal of the mega model is to assess and predict interactions between terrestrial ecosystems and many climate-related processes. Note that the terrestrial ecosystem model (Figure 7.7) is embedded within the mega model. The numbers show the dimension of the surface area grids used to assemble the model (units are degrees latitude by degrees longitude). (From Xiao, S., D.W. Kicklighter, J.M. Melillo, A.D. McGuire, P.H. Stone, and A.P. Sokolov. 1997. Linking a global terrestrial biogeochemical model and a 2-dimensional climate model: implications for the global carbon budget. *Tellus* **49B**:18–37. With permission from Blackwell Publishing, Oxford, UK.)

(2007, 2010), Piao et al. (2013), Le Quéré et al. (2013), and Zaehle and Dalmonech (2011).

Keeping in mind the above overview of environmental compartments, elemental pools, and fluxes between pools, we now turn to the detailed microbiological processes that drive many of the fluxes.

7.3 THE "STUFF OF LIFE": IDENTIFYING THE POOLS OF BIOSPHERE MATERIALS WHOSE MICROBIOLOGICAL TRANSFORMATIONS DRIVE THE BIOGEOCHEMICAL CYCLES

Conceptually, the "stuff of life" is two things: (i) it is the vital biomass, the bodies of functioning creatures (e.g., viruses, *Bacteria*, *Archaea*, fungi, protozoa, nematodes, insects, plants, animals, humans; see Sections 4.5 and 5.5 to 5.10) presently alive on Earth and (ii) it is the substances (light and thermodynamically unstable mixtures of materials; see Sections 3.6 to 3.11) that fuel biochemical reactions used to keep the creatures alive and well. From the viewpoint of microbiology, *the selective pressures that have driven evolution operate via specific organic and inorganic compounds*. These are the resources of our planet – that have been (and continue to be – day-by-day, minute-by-minute) utilized for catabolic and anabolic purposes. Particular compounds occur in particular geochemical contexts. Remember that evolution acts at the level of mutations in DNA and their manifestation as altered enzyme recognition, specificity, and function.

Information in Table 7.4 provides a listing of many of the substances in the biosphere that drive microbial biogeochemistry. The listing is not comprehensive, but it is representative of biogeochemically important pools and processes. The substances are sorted by dominant elemental cycle (C, S, N, P, O, H, Fe, O, and Hg.) and type (organic or inorganic). Included in the table is an overview of the sources of the compounds, mechanisms of biochemical utilization, and examples of key biota involved in substance utilization. The organic compounds, shown in columns 2 and 3 of Table 7.4, are, in essence, a compositional analysis of the major biomolecules in current life forms or what they have become (peat, humic substances, kerogen, petroleum, coal). The inorganic compounds (also columns 2 and 3 of Table 7.4) are gases or minerals in soil, sediments, waters, and/or the atmosphere that participate in metabolic processes as electron donors, as electron acceptors, as building blocks of biomolecules, or as potential toxicants (in the case of Hg). Each of the individual entries in Table 7.4 has its own mechanism of synthesis (formation) and consumption (destruction). The size of the pools of these materials found in soils, sediments, and waters across the globe reflects the dynamic balance between paths of formation versus destruction. Cellulose, elemental sulfur, proteins, nitrate, phosphates, hydrogen gas, Fe^{2}+, oxygen gas, even metallic mercury – all of these (and more) are subject to microbially mediated processes. Also, each process occurs under specific conditions and is catalyzed by specific populations. For example, mercury undergoes methylation reactions (final entries of Table 7.4) that are thought to be fortuitously (vitamin B_{12} donation of a methyl group) mediated by sulfate- and iron-reducing microorganisms. The extent of methyl-mercury formation in a given locale is, thus, governed indirectly by cycling rates of carbon (electron donor), iron, and sulfur.

Table 7.4

Selected compounds in the biosphere that participate in major microbial biogeochemical processes. (Compiled from Zabel and Morrell, 1992; Buist et al., 1995; Lengeler et al., 1999; Ehrlich, 2002; Zahrl et al., 2005; Metcalf et al., 2012, Su et al., 2011; Martinez et al., 2011; Karl et al., 2008; Stemmler et al., 2006; Kertesz, 2000; McGrath et al., 2013; Denger et al., 2014; Dyhrman et al., 2007; Schink and Friedrich, 2000)

Dominant elemental cycle	Type of compound	Compounds in reservoirs	Production and location of compounds	Key process for compound utilization*	Key organisms active in utilization
Carbon (C)	Organic (live and dead biomass)	Cellulose	Plants; in soils, sediments, water	Hydrolysis via cellulases; glucosidases, cellulosomes; and respiration of subunits	Fungi, prokaryotes
		Lignin	Plants; in soils, sediments, water	Enzymatic combustion via Fe-ligninase, Mn-peroxidase,Cu-laccase	Fungi
		Hemicellulose	Plants; in soils, sediments, water	Hydrolysis via hemicellulase; and respiration of subunits	Fungi, prokaryotes
		Starch	Plants; in soils, sediments, water	Hydrolysis via amylases; and respiration of subunits	All life
		Chitin	Fungi, insects, marine zooplankton; in soils, sediments, water	Hydrolysis via chitinases and *N*-acetyl glucosaminidase; and respiration of subunits	Prokaryotes, fungi
		Peptidoglycan	Prokaryotes; in soils, sediments, water	Hydrolysis via peptidoglycan hydrolase, lytic transglycosylase, proteases; and respiration of subunits	Prokaryotes, fungi
		Lipids	All life; in soils, sediments, water	Hydrolysis via lipase; and respiration of subunits	All life
		Protein	All life; in soils, sediments, water	Hydrolysis via proteases; and respiration of subunits	All life
		Nucleic acids	All life; in soils, sediments, water	Hydrolysis via nucleases; and respiration of subunits	All life

Table 7.4 *Continued*

Dominant elemental cycle	Type of compound	Compounds in reservoirs	Production and location of compounds	Key process for compound utilization*	Key organisms active in utilization
		Humic substances	Traditionally depicted as formed via random biotic and abiotic synthesis in soils, sediments; but more recently recognized to be spatially distinct mixed aggregates of decomposition products that persist in soil due to the protection by the soil matrix.	Prokaryotic and fungal enzymes, physical and chemical weathering	Prokaryotic heterotrophs
		Peat, kerogen, coal	Diagenetic processes in shallow to deep geologic formations	Resistant	–
		Petroleum	Diagenetic processes in deep geologic formations; surface catalyzed reduction of CO_2	Hydroxylation by oxygenase enzymes, β-oxidation of alkanes, dioxygenase cleavage of aromatic rings; anaerobic activation to form conjugated organic acids leading to reduction and hydrolysis; and respiration	Aerobic and anaerobic prokaryotes, and fungi
Carbon (C)	Inorganic	CO_2	Acidification of carbonate minerals, biotic respiration; in atmospheric pool	Final electron acceptor; photosynthesis	Methanogenic *Archaea*; plants, algae, chemolithotrophic prokaryotes
		CH_4	Diagenetic processes in geologic formations; surface-catalyzed reduction of CO_2; microbial methanogenesis in anaerobic microbial communities (three anaerobic pathways: from acetate; from $H_2 + CO_2$; from methanol and methylamines); aerobic (C–P lyase) attack of methylphosphonate; in atmospheric pool, peteroleum deposits, and in gas hydrate "clathrate" deposits in ocean floor	Used as carbon and energy source (methanotrophy)	Aerobic and anaerobic prokaryotic methanotrophs; anaerobic methanotrophic *Archaea* (ANME) operate as obligate partners with sulfate reducers or Fe- or Mn-reducers; ANME may also work alone, reducing sulfate to disulphide; *Methoxymirabilis* oxidizes methane with nitrite as the electron accept while producing O_2 as a critical intermediate

		CO	Lignin catabolism, fossil fuel combustion; in atmosphere	Used as electron donor for ATP generation	Carboxydotrophic, acetogenic, and methanogenic prokaryotes
Sulfur (S)	Organic (live and dead biomass)	Protein (especially methionine and cysteine; metallo enzymes)	All life; in soils, sediment, water	Hydrolysis via proteases; and respiration of subunits	All life
		Humic substances	Random biotic and abiotic synthesis in soils, sediments (see alternative origin above, as described for organic C)	Prokaryotic and fungal enzymes, physical and chemical weathering	Heterotrophic prokaryotes, fungi
		Sulfonates (R–SO_2O–) and sulfate esters	Various biosynthetic pathways in plants algae, microorganisms; mammals; large component of organic S in soils and ocean waters	As nutritional S source	Heterotrophic microorganisms in soil and the oceans
		Sulfoquinovose (SQ)	6-Deoxy-6-sulfo-D-glucopyranose (SQ) is the polar headgroup of sulfolipids in many plant-cell membranes; also synthesized by some prokaryotes	Carbon and energy source, degraded in bacteria to 2,3-hydroxypropane-1-sulfonate, which is then mineralized to sulfate and CO_2	Heterotrophic microorganisms in soil, marine, and freshwater habitats; microorganisms in soil
	Inorganic	Sulfate	Salt dissolution, sulfide oxidation, elemental sulfur oxidation; in soils, sediment, water (especially oceans)	Used as final electron acceptor in dissimilatory sulfate reduction, reduced during sulfate reduction by ATP sulfurylase enzyme	Heterotrophic anaerobic prokaryotes
		Thiosulfate	Chemical oxidation of sulfur minerals; in soils, sediment, waters	As electron acceptor, reduced to sulfide As electron donor, oxidized to sulfate	Sulfate-reducing bacteria Sulfur-oxidizing chemo-lithotrophic bacteria
		Dimethyl sulfoxide (DMSO)	Oxidation of DMS; in marine systems	Reduced as electron acceptor to DMS; used as electron donor, oxidized to sulfate	Prokaryotic anaerobic heterotrophs, aerobic heterotrophs

Table 7.4 *Continued*

Dominant elemental cycle	Type of compound	Compounds in reservoirs	Production and location of compounds	Key process for compound utilization*	Key organisms active in utilization
		Elemental S	Sulfide oxidation and sulfate reduction; in soils, sediment, water	Electron acceptor (reduced to sulfide) and electron donor (oxidized to sulfate) for ATP generation and for phototrophic CO_2 reduction (oxidized to sulfate)	Anaerobic prokaryotes, chemolithoautotrophs, phototrophic S bacteria
		Dimethyl sulfide (DMS)	Released from decaying marine algae, reduction of DMSO; in marine systems	Electron donor for ATP generation with oxygen, nitrate and sulfate as electron acceptor; and for CO_2 reduction and methanogenesis	Chemoorganotrophs, chemolithotrophs, phototrophs, methanogens
		Hydrogen sulfide, mineral sulfides	Geothermal gases, sulfate reduction; in geologic deposits, sulfate-rich anaerobic sediments	Electron donor for ATP generation and for phototrophic CO_2 reduction	Chemolithoautotrophs, phototrophic S bacteria
Nitrogen (N)	Organic (live and dead biomass)	Protein	All life; in soils, sediments, water	Hydrolysis via proteases; and respiration of subunits	Prokaryotic and eukaryotic heterotrophs
		Humic substances	Random biotic and abiotic synthesis in soils, sediments (see alternative origin above, as described for organic C)	Prokaryotic and fungal enzymes, physical and chemical weathering	Prokaryotic heterotrophs, fungi
		Trimethylamine *N*-oxide (TMAO)	Nitrogenous excretion by fish	Used as electron acceptor, reduced to trimethylamine	Prokaryotic anaerobes
		Urea $[CO(NH_2)_2]$	Industrial fertilizer; mammalian metabolism; decomposition of organic N compounds in soil, water, and atmosphere	Chemical and enzymatic (via urease) hydrolysis to ammonia and CO_2; nutritional N source	Prokaryotic and eukaryotic microorganisms

	Inorganic	NH_3	Dinitrogen fixation by prokaryotes; ammonification from amino acids; dissimilatory nitrate reduction to ammonia; in soils, sediment, water; deamination of proteins	Assimilation†: nutrient uptake	Plants, microorganisms
				Dissimilation‡: used as electron donor for ATP generation; nitrification, anammox	Prokaryotic chemolithotrophs (nitrifiers, anammox)
		NO_3^-	Nitrification; in soils, sediment, water	Assimilation: nutrient uptake	N-assimilation pathways in plants, fungi, prokaryotes
				Dissimilation: used as electron acceptor for ATP generation; denitrification to N_2 or dissimilatory nitrate reduction to NH_4^+	Nitrate-reducing prokaryotes
		NO_2^-	Nitrification and denitrification; in soils, sediment, water	Used as electron acceptor reactant in anammox; also reduced to NO in denitrification; conversion to atmospheric HONO at low pH in soil;	Anammox and denitrifying prokaryotes
				oxidized to nitrate in nitrification	Nitrifying prokaryotes
		NO	Intermediary metabolite in denitrification and annamox; in soils, sediment, water	Used as electron acceptor (reduced to N_2O)	Prokaryotic denitrifiers (see Table 7.6 and Box 7.7)
		N_2O	Intermediary metabolite in denitrification; in soils, sediment, water	Used as electron acceptor (reduced to N_2)	Prokaryotic denitrifiers (see Table 7.6 and Box 7.7)
		N_2	Metabolic endproduct of denitrification and anammox; in soils, sediment, water; atmospheric pool	Reduced to NH_3 during nitrogen fixation by nitrogenase	Nitrogen-fixing prokaryotes
Phosphorus (P)	Organic (live or dead biomass)	Phospholipids	All life; in soils, sediment, water	Hydrolysis, assimilation, catabolism	All life
		Nucleic acids	All life; in soils, sediment, water	Hydrolysis, assimilation, catabolism	All life

Table 7.4 *Continued*

Dominant elemental cycle	Type of compound	Compounds in reservoirs	Production and location of compounds	Key process for compound utilization*	Key organisms active in utilization
		ATP	All life; in soils, sediment, water	Catabolism	All life
		Phytin (inositol hexaphosphate)	P-storage compound, especially in plant tissue	Phytase enzyme hydrolyzes C–O–P bonds	Microorganisms; plant and animal tissues
		Phosphonates (direct C–P bond; e.g., methylphosphonate $CH_3(PO_3H_2)$)	Biosynthesis in marine microorganisms (*Nitrosopumilus*); ocean waters	C–P bond cleaved and reduced by C–P lyase enzyme	Heterotrophs and autotrophs requiring a nutritional P source
	Inorganic	Mineral phosphates	Mineral reservoirs; mineralization of biomass	Uptake, assimilation	All life
		Phosphite (e.g., PO_3^{3-})	Geochemical reduction of phosphate	Oxidized to phosphate and assimilated as P-source in biomass; used as electron donor for growth and as a P-source	*Prochlorococcus*, *Desulfotignum*; *Phosphitoxidans*
Hydrogen (H)	Inorganic	H_2 gas	Geothermal formation; anaerobic food chains in sediments and in animal guts; atmospheric pool	Electron donor for ATP generation for aerobes and anaerobes	Chemolithotrophic prokaryotes (e.g., aerobic hydrogen oxidizers, methanogens)
Iron (Fe)	Organic (live and dead biomass)	Heme, metalloenzymes	Anabolism in all life	Catabolism	All life
	Inorganic	Fe^{2+} (aq) and in minerals	Minerals; reduction of Fe^{3+}; in soils, sediment, water	Biotic and abiotic (O_2) oxidation of Fe^{2+} to Fe^{3+} Electron donor for chemolithoautotrophs using O_2 and NH_3^- as final electron acceptors	Iron-oxidizing bacteria, which include heterotrophs (e.g., *Leptothrix*) and autotrophs (e.g., *Thiobacillus ferroxidans*)

				Electron donor for anoxygenic photosynthetic bacteria	Purple, nonsulfur bacteria
		Fe^{3+} (e.g. Fe_2O_3, $Fe(OH)_3$)	Minerals, chemical and microbial oxidation of Fe^{2+}; in soils, sediment, water	Anaerobic final electron acceptor in respiratory ATP generation	Anaerobic prokaryotes (e.g., *Geobacter*)
Oxygen (O_2)	Inorganic	O_2 gas	Oxygenic photosynthesis; chlorate respiration; detoxification of intracellular reactive oxygen species (ROS); nitrite-driven anaerobic methane oxidation (via nitric oxide dismutase); in atmosphere	Aerobic respiration; Incorporation of oxygen into organic compounds via oxygenase enzymes	Aerobic organisms (*Bacteria, Archaea, Eucaryoe*)
Mercury (Hg)	Organic	CH_3Hg+, $(CH_3)_2Hg$	Methylation predominantly by sulfate-reducing and Fe-reducing bacteria in anaerobic sediments	Demethylation and reduction to Hg^{2+} and Hg^0	UV light, prokaryotic heterotrophs, methanogens, fungi (see Sections 8.3 and 8.6)
	Inorganic	Hg^0	Minerals, reduction and photoreduction (in the presence of dissolved organic carbon) of Hg^{2+}, demethylation of methyl-Hg	Methylation by vitamin B_{12}	Sulfate-reducing and Fe-reducing prokaryotes, fungi
		Hg^{2+}	Minerals, photo-oxidation of Hg^0; in sediments	Detoxification via reduction; methylation	Prokaryotes with *mer* genes; sulfate-reducing and Fe-reducing prokaryotes

*Major biological mechanism(s) of compound utilization include enzymatic steps in biogeochemical cycles.
†Assimilation: nutrient uptake – incorporated into cellular components and biomass.
‡Dissimilation: used as final electron acceptor – reduced and released as metabolic waste; not incorporated into cell biomass.

Hydrolytic breakdown of polymers

Most of the organic compounds shown in column 3 of Table 7.4 are the products of cellular biosynthetic reactions creating essential structural polymers (e.g., cellulose, lignin, lipids, chitin, protein; as membranes, cell walls, exoskeletons, muscle tissue, etc.; see Section 3.9). A key step in the recycling of these high molecular weight (insoluble, often particulate) materials is conversion, via hydrolysis, to constituent monomers that can be transported to the interior of microbial cells to fuel catabolic and/or anabolic reactions. "Hydrolysis" is, by definition, the nucleophilic attack of a water molecule across a C–C bond: H is added to one side and OH to the other as two molecules are released from the site of bond cleavage. Box 7.3 describes the major biopolymers of the biosphere and their metabolism.

Box 7.3

Major polymers of the biosphere and their metabolism

Biopolymer	Monomer	Number of monomers	Glucosidic and other bonds
Starch	Glucose	100–1000	α-1,4 and α-1,6
Hemicellulose	Xylose, glucose, mannose, galactose, uronic acids	~200	β-1,4 and cross links
Cellulose	Glucose	3000–26,000	β-1,4
Lignin	Phenyl propane units (*p*-hydroxycinnamyl alcohols)	~10,000	Random carbon–carbon and ether linkages
Chitin	*N*-acetyl glucosamine	~10,000	β-1,4
Peptidoglycan	*N*-acetylglucosamine, *N*-acetyl muramic acid, amino acids	4,000	β-1,4 tetrapeptide cross links

Several biochemical properties of the first six biopolymers in Table 7.4 are shown above. Constituent monomers, chain length, and the type of chemical bonds between monomers have major effects on biopolymer properties.

Starch

The most common storage material of plants is composed of glucose monomers, linked in a branching pattern that connects the carbon number 1 of a given glucose molecule to either carbon number 4 or number 6 of an adjacent glucose molecule. Thus, the bonds between the two sugars are "1,4-" and "1,6-"glucosidic bonds. The "α" in column 4 (above) refers to the geometry of the C–O–C linkage between molecules. Starch molecules composed of up to 1000 monomers are readily susceptible to hydrolysis by amylosaccharide (or "amylase") enzymes that are widespread among organisms (Lengeler et al., 1999).

Box 7.3 *Continued*

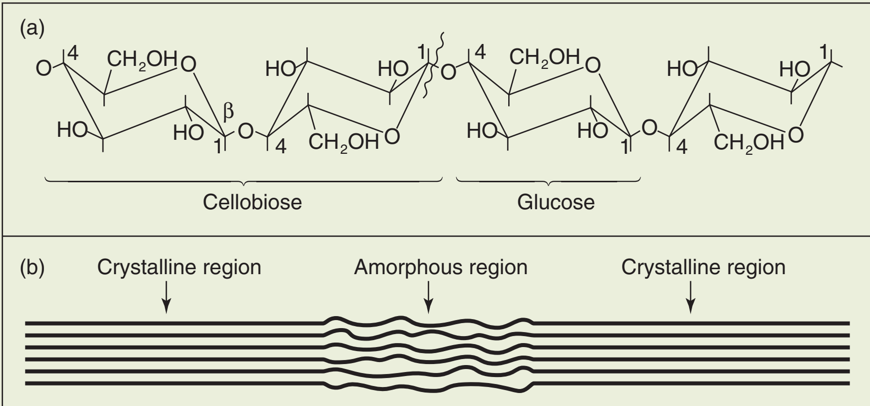


Figure 1 Structure of cellulose: (a) β-glucosidic bonds and (b) schematic structure of a cellulose fibril. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 9.3. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

Cellulose

Though cellulose is also formed from glucose units with bonds between carbons number 1 and 4, the geometry of the C–O–C bond linkage is in the opposite (β) configuration from those in starch. The β configuration confers high tensile strength and causes a 180° rotation of each glucose molecule relative to its neighbor (Figure 1). In addition, the combination of very long chains (~10,000 monomers) and hydrogen bonding between adjacent chains causes the polymers to form fibrils that are rigid, insoluble, and crystalline. These properties render cellulose susceptible to attack by only a highly specialized set of enzymes encoded by a limited set of bacteria and fungi.

Plant tissue

Plant tissue, especially wood, is a major pool of organic carbon in the biosphere (see Sections 7.4 and 7.6). Biochemically, wood is termed "lignocellulose" (Hammel, 1997; Deobold and Crawford, 2002) because cellulose and lignin are in intimate contact. Functionally, lignin adds strength and rigidity to cellulose fibrils. During tree growth, wood biosynthetic reactions encrust the cellulose fibril within a matrix of lignin monomers (benzene rings with three-carbon side chains, known as "phenyl propane" units). Peroxidase and laccase enzymes contribute to lignin biosynthesis by activating the lignin monomers as free radicals and they polymerize randomly and spontaneously (Hammel, 1997). Thus, lignin (Figure 2) in trees bears a close resemblance to, and is a precursor of, humic substances in soil. Clearly, as a habitat for microbial growth, wood presents many challenging characteristics: it is insoluble, crystalline, dry, of low nutrient (low proportions of N, P, Fe, etc.), and a structurally integrated matrix of cellulose and lignin (with other polysaccharides). Fungi, particularly *Basidiomycetes* and *Ascomycetes* (see Section 5.6), are considered the major players in lignocellulose decomposition (Zabel and Morrell, 1992; Hammel, 1997). Though bacteria

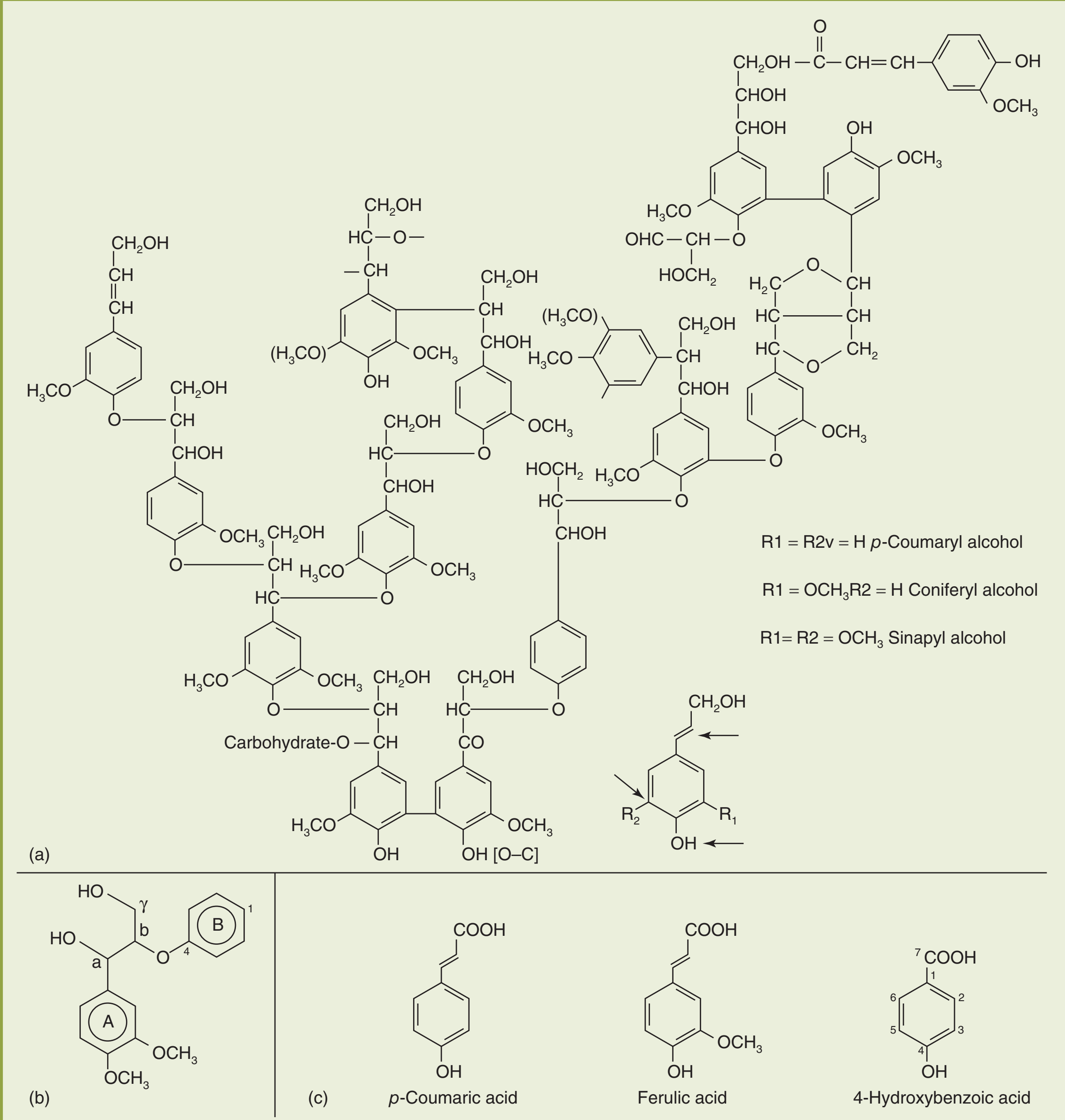


Figure 2 Lignin structure and its constituents. (a) The three precursor alcohols of lignin are shown on the lower right. One electron oxidation of these lignin building blocks and subsequent polymerization reactions produce the three-dimensional lignin structure shown. The arrows indicate the position of intermonomeric bonds in lignin, including arylether, biphenyl, and diphenylether bonds. (b) Lignin model compound with β-O-4 ether linkage. (c) Aromatic acids found in lignin–carbohydrate linkages. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 9.9. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

Box 7.3 *Continued*

are also well adapted to carry out cellulose metabolism in some contexts (especially anaerobically, see below), the filamentous form of fungi and their unique ligninase enzyme system set them apart (Hammel, 1997).

Natural history of wood decay in forests

There are three outward types of wood decomposition: white rot, brown rot, and soft rot. These three designations are based upon the visual and textural qualities of the wood (an initial mixture of white (cellulosic) and brown (ligninic) tissues; Zabel and Morrell, 1992). After *white rot* fungi (largely *Basidiomycetes* and *Ascomycetes*; Hammel, 1997) have completed their work, the remaining logs are white because virtually all the lignin has been removed, leaving only remnants of the original cellulose. *Brown rot* fungi decay the cellulose preferentially – leaving behind the majority of the original brown lignin-rich tissue. The characteristic of *soft rot* is that it occurs often in water-saturated (though aerobic) habitats – leaving the woody tissue porous and structurally weak. Soft rot fungi (*Ascomycetes*) attack the polysaccharide components preferentially, but also decompose lignin (Hammel, 1997); their enzymatic degradative mechanisms are not yet well characterized.

Cellulose decomposition

Cellulose decomposition is an extracellular process that releases the glucose subunits as carbon and energy sources to the active microbial (largely fungal) populations. The fungal hyphae deliver three functionally distinctive, yet highly cooperative, cellulase enzymes to an eroding cellulosic surface: (i) endoglucanases, which randomly hydrolyze 1,4-bonds internal to the long cellulose molecules; (ii) exoglucanases, which sequentially cleave two-unit (cellobiose) molecules from one end of the chains freed by the endoglucanases; and (iii) β-glucosidases, which hydrolyze cellobiose (and other low molecular weight sugar fragments) into glucose molecules (Leschine, 1995; Hammel, 1997; Lengeler et al., 1999; Igarashi et al., 2011).

Cellulosomes

While the basic three-step mechanism of cellulose hydrolysis holds for all active microorganisms (fungi and bacteria), each possesses a refined cluster of cellulase enzymes that vary in the *endo*- versus *exo*- mode of attack and recognition of crystalline versus amorphous regions in cellulose molecules (see cellulose structure, in Figure 1). One unique metabolic adaptation in anaerobic cellulolytic bacteria and fungi that operate in submerged aquatic habitats is the *cellulosome* (Leschine, 1995; Bayer et al., 2004; Doi and Kosugi, 2004). Cellulosomes are organelles (high molecular weight, e.g., 65,000 kDa) – multienzyme complexes situated on the exterior of cells. Electron micrographs and other procedures reveal them as "extracellular protuberances" (Doi and Kosugi, 2004) that consist of a three-dimensional framework (scaffold) that arranges as many as 35 different enzymes that both bind to and hydrolyze the cellulose and related polysaccharides in plant cell walls. The genes that encode cellulosome proteins and the intricacies of gene resolution have begun to be explored (Doi and Kosugi, 2004).

Lignin

Lignin is not metabolized as a carbon and energy source – instead lignin is degraded by ligninolytic fungi to expose the more digestible polysaccharides of wood so that these can be cleaved by fungal cellulases and hemicellulases (Hammel, 1997). Ligninolysis is not yet fully understood (Hammel, 1997; Leonowicz et al., 1999; Hofrichter, 2002), though many rudimentary aspects have been described. The random, free-radical catalyzed synthesis of lignin renders it resistant to hydrolytic cleavage. Given the chemical recalcitrance of lignin, white rot fungi have evolved a novel biochemical approach to degrade it: random free-radical oxidation (combustion) reactions involving molecular oxygen. Lignin peroxidase, manganese peroxidase, and copper-containing laccase enzyme systems utilize very strong oxidants (ferric heme, Mn^{3+}, and Cu^{2+}, respectively) that attack C–C bonds in the lignin, removing electrons (Figure 3). The destabilized intermediates undergo ring-cleavage and ether-cleavage reactions that release low molecular weight lignin “structural monomers”. In order for the delignification process to persist, laccase, ligninase peroxidase, and manganese peroxide enzymes that have stolen electrons from the lignin structure must be reoxidized. Extracellular H_2O_2 (a strong oxidant) fulfills this role: H_2O_2 is generated by the reaction between O_2 gas and a commonly excreted fungal compound, glyoxal (Figure 3). Overall, then, the three enzymes responsible for destroying lignin shuttle electrons from lignin, itself, to H_2O_2.

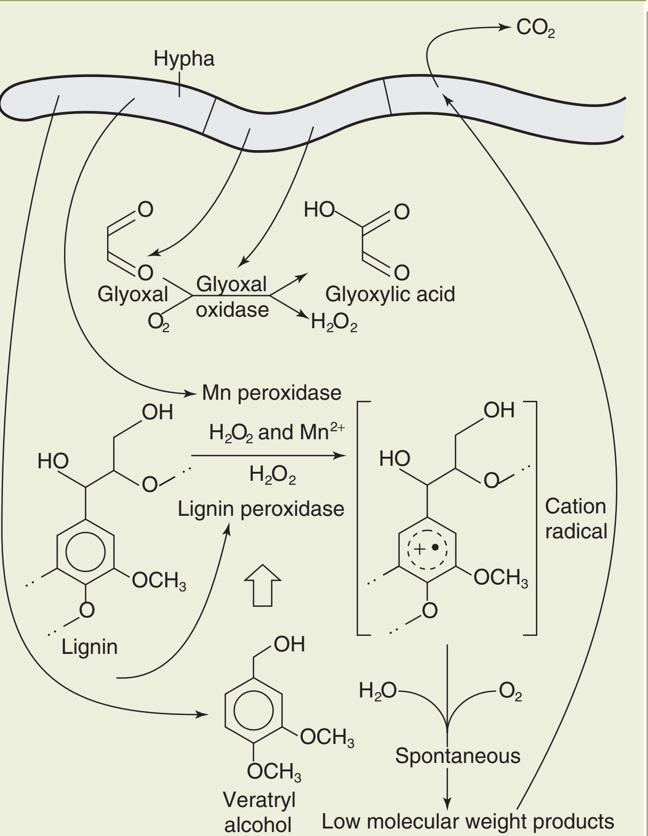


Figure 3 The ligninolytic system of the white rot fungus, *Phanerochaete chrysosporium*. See the above box text for details. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 9.10. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

Biodegradability and recalcitrance of organic compounds

Prevailing wisdom in environmental microbiology deems that all naturally occurring biosynthetic compounds are biodegradable (Alexander, 1973, 1999; Wackett and Herschberger, 2001). Indeed, the majority of

organic materials (e.g., carbon-containing compounds excluding graphite, CO, and CO_2) shown in Table 7.4 are useful carbon and energy sources or nutrients for heterotrophic prokaryotes and fungi. Some of the biopolymers (e.g., starch, protein) are useful to higher eukaryotes as well. The compounds in Table 7.4 that are resistant to enzymatic attack and metabolism are: coal, kerogen, peat, and humic substances. Both *kerogen* and *coal* are fossilized, highly metamorphosed, mineral-like forms of carbon found in geologic deposits (coal is in massive deposits, while kerogen is highly dispersed throughout sedimentary rocks, such as shales). Peat, the geologic predecessor of coal, is accumulated plant biomass (especially *Sphagnum*-type mosses) in wetlands that are often acidic. The combination of anaerobic conditions, cellulosic/ligninic material, accrual of organic-acid fermentation products, and low pH leads to preservation of peat materials (including the remains of humans retrieved from ancient peat bogs!).

Like coal and kerogen, *humic substances* (Table 7.4) are a globally significant pool of organic carbon that resist microbiological attack. However, unlike coal and kerogen, humic substances do not form in the presence of high temperature and pressure. The traditional view of humic substances has, for decades, been that they are high molecular weight, "polymer-like" networks of molecules composed of plant-derived and microbially derived random subunits linked (synthesized) by extracellular enzymatic and free-radical reactions into random covalent linkages (Figure 7.9; Stevenson, 1994). Sutton and Sposito (2005) have recently developed a new argument that depicts humic substances as "collections of diverse, relatively low-molecular-mass compounds forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds". Evidence for the new view was derived from physical, molecular, and spectroscopic data showing: (i) that humic substances are aggregates of micelle-like particles (400–800 nm in size) held together by hydrophic associations; (ii) the chemical moieties in these particles are diverse and derived from recognizable low molecular weight biomolecules such as lipids, lignin, carbohydrates, and protein; and (iii) that the major functional organic moieties (amide groups), thought to participate in forming the traditional polymer-like model of humic substances, are largely unoccupied. Recent reviews by both Schmidt et al. (2011) and Kleber and Johnson (2010) reinforce the emerging understanding that humic substances occur at the nano- and microscale as mixtures of recognizable biomolecules that are protected from enzymatic attack (decomposition) by their physical relationships (e.g., sorption, hydrophobicity, inaccessibility) to the soil physical matrix (Lehman et al., 2007). There are still many unanswered questions about humic substances: the dark, refractory, heterogeneous organic compounds that are crucial for creating agriculturally desirable soil properties and that are among the most widely distributed organic materials in terrestrial habitats.

Figure 7.9 Schematic (traditional) model of humic acid. Included in the structure are molecular remnants of amino acids and sugars, showing possible associations with inorganic compounds including silica (Si-O) moieties at the surface of clay and other minerals. See text for details and an alternative, more recent model that portrays soil organic matter as microscale aggregates of recognizable decomposition products protected from additional microbial attack by the physical soil matrix. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 31.11. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

Petroleum reservoirs (or crude oil, see Table 7.4) represent another form of metamorphosed organic matter (plants and algal lipids). Time, heat, and pressure caused diagenetic alterations in the molecular structures of the original biomass so that liquid and gaseous hydrocarbons migrated until they accumulated in porous rocks, forming an oil field. While petroleum hydrocarbons are both "naturally occurring" and "biodegradable", the optimal conditions (enzymes, microorganisms, physiological reactions) for growth of heterotrophic hydrocarbon-degrading prokaryotes and some fungi are restricted. If this were not the case, geological petroleum reservoirs would not exist.

Two major classes of hydrocarbons are straight-chain alkanes and benzene-containing aromatic compounds (see also Section 8.3 and Boxes 8.5 and 8.7). By definition, "hydrocarbons" consist almost exclusively of carbon and hydrogen atoms. Oxygen was eliminated from the organic precursor materials during oil diagenesis; only small amounts of nitrogen, sulfur, and trace metals remain (and are released during industrial combustion of petroleum fuels). Though highly reduced and thermodynamically unstable in the presence of potential electron-accepting oxidants (e.g., CO_2, SO_4^{2-} Fe^{3+}, Mn^{4+}, NO_3^- and O_2; see Section 3.8 and Figure 7.10), breakage of C–C bonds in petroleum molecules is a specialized metabolic task. When oxygen is a reactant, petroleum biodegradation is most rapid. Oxygenase enzymes add molecular oxygen to the molecule. This process can be catalyzed by a

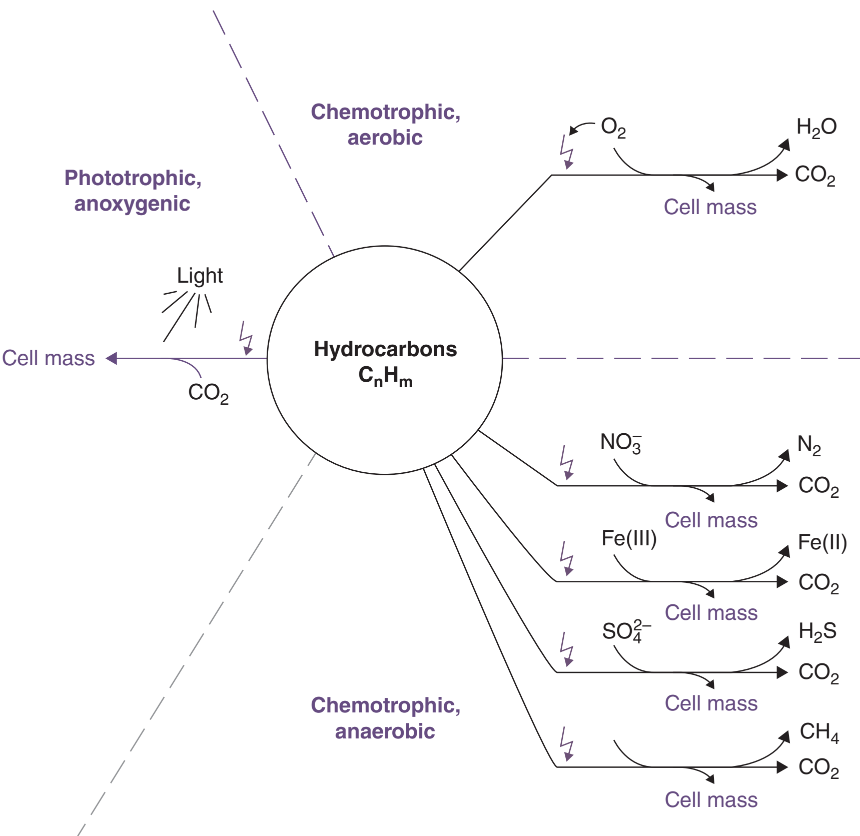


Figure 7.10 Experimentally verified possibilities for the microbial utilization of hydrocarbons. In all chemotrophic reactions, a part of the hydrocarbon is oxidized for energy conservation (catabolism) and another part is assimilated into cell mass. In the long-established aerobic oxidation of hydrocarbons (upper right), oxygen is not only the terminal electron acceptor but is also needed for substrate activation (oxygenase reactions). The anaerobic pathways involve novel hydrocarbon activation mechanisms that differ completely from the aerobic mechanisms. Jagged arrows indicate hydrocarbon activation. (Reprinted from Widdel, F. and R. Rabus. 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **12**:259–276. Copyright 2001, with permission from Elsevier.)

monooxygenase enzyme (forming one C–OH bond) or a dioxygenase enzyme (forming two adjacent C–OH bonds). Molecules with C–OH bonds are alcohols. Oxygen incorporation into hydrocarbon molecules requires reducing power (e.g., often in the form of NAD(P)H – a molecule related to electron carriers used in respiration; see Section 3.9 and Box 3.8) that converts oxygen from the zero oxidation state to −2 (Alexander, 1999; Lengeler et al., 1999; Van Hamme et al., 2003).

If the newly hydroxylated molecule was an alkane, it is enroute to conversion into a carboxylic acid and reactions, termed β-oxidation (Figure 7.11). Beta-oxidation is a six-step series that: (1) links the carboxylic acid to coenzyme A (CoA, composed of pantothenic acid and β-mercaptoethylamine) via a thioesther bond; (2) dehydrogenates a C–C bond in the "beta position", two carbons in from the original terminus; (3) adds water to that bond, resulting in hydroxylation; (4) oxidizes the new hydroxyl to a keto group; (5) cleaves off a two-carbon moiety, acetyl-CoA, from the hydrocarbon chain while generating an SCoA-linked hydrocarbon molecule two carbons shorter than the original; and (6) repeats steps 1 to 5 until enzymatic digestion is complete (Lengeler et al., 1999). If the newly hydyroxylated molecule was originally an aromatic (benzene) ring, then aerobic metabolism proceeds via: (i) oxidative ring cleavage by dioxygenases and (ii) further cleavage of C–C bonds, creating noncyclic, nonaromatic ring fission products, such as pyruvate, that feed into central metabolic pathways such as the Krebs citric acid cycle (bottom of Figure 7.12; Lengeler et al., 1999; see also Section 3.9 and Box 3.8).

Though anaerobic metabolism of petroleum hydrocarbons is kinetically and energetically far less favorable than in the presence of oxygen, some aspects of the process have been well characterized (see Figure 7.10; Spormann and Widdel, 2000; Widdel

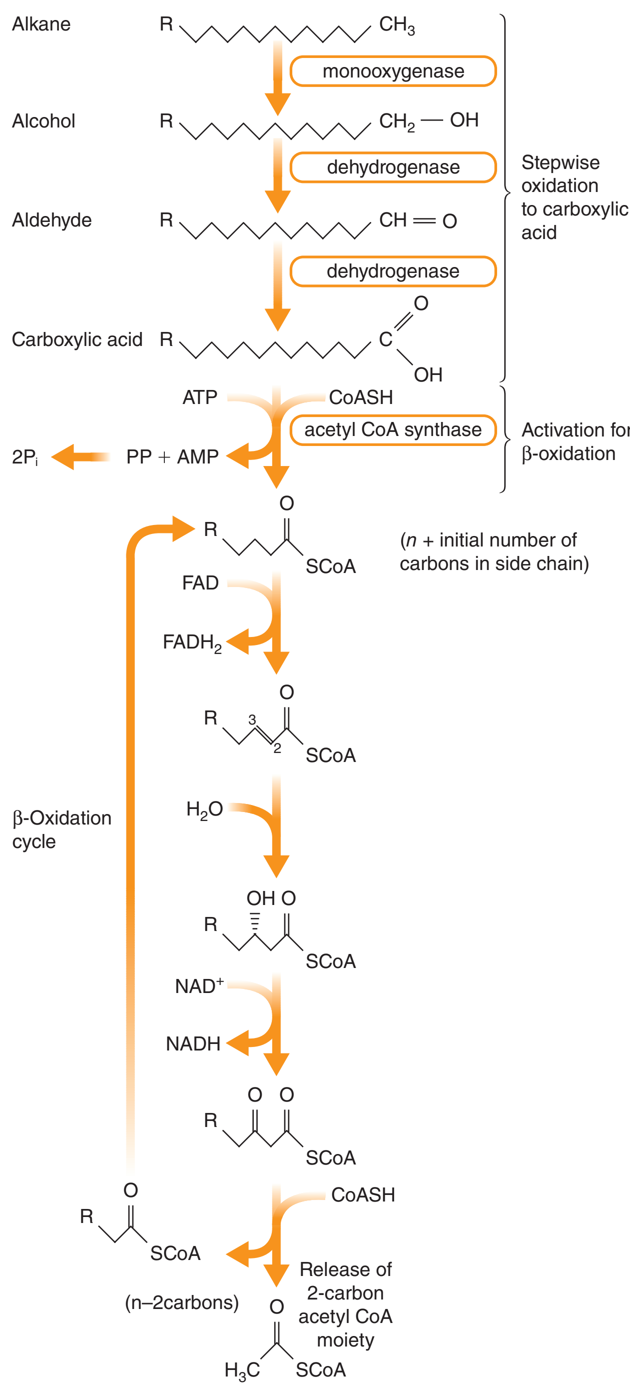


Figure 7.11 Pathway for aerobic metabolism of alkane petroleum hydrocarbons, featuring stepwise terminal oxidation to a carboxylic acid and subsequent β-oxidation. See text for details. Pi, inorganic phosphate; PP, pyrophosphate; SCoA, thioester-linked acetyl CoA. (Modified from Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 9.37. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

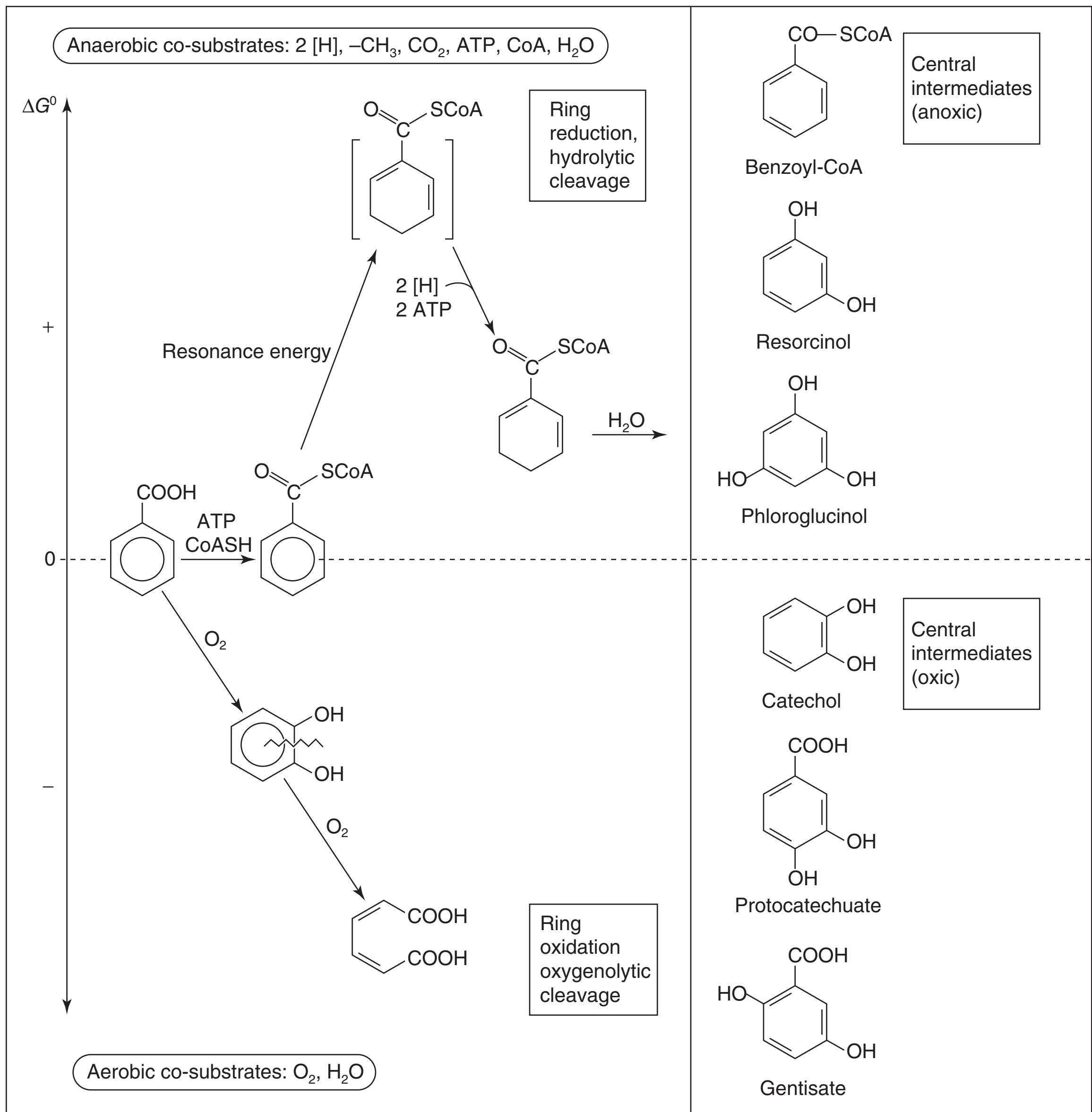


Figure 7.12 Comparison of aerobic (oxygen-dependent) and anaerobic (anoxic) metabolism of aromatic compounds. Shown are the co-substrates used and several key intermediary metabolites. Under oxic conditions, benzoate is converted, for example, to catechol, and the aromatic ring is oxygenolytically cleaved. Under anoxic conditions, the high resonance energy does not allow direct reduction of the aromatic ring. Rather, ring destabilization is achieved by forming the coenzyme A (CoA) thioester, which requires ATP. Then the ring is reduced at the cost of another ATP and finally the ring is opened hydrolytically. See text for additional details. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 9.29. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

and Rabus, 2001; So et al., 2003; Heider, 2007; Fuchs et al., 2011; Holmes et al., 2012). Alkanes are initially anaerobically attacked by enzymes that convert alkanes to organic acids via addition of the four-carbon dicarboxylic acid, fumarate. Though these and subsequent biochemical steps are still being actively researched, it is likely that β-oxidation-type reactions then proceed (as described above, Lengeler et al., 1999). Without molecular oxygen as a reactant to destabilize the benzene ring of aromatic compounds (top of Figure 7.12), anaerobic microorganisms, instead, appear to use water, bicarbonate, ATP, CoA thioesters, methyl donors, and reduced and oxidized coenzymes to sequentially reduce and then hydrolytically cleave aromatic structures to low molecular weight intermediary metabolites that funnel into central biosynthetic and catabolic (respiratory) pathways (Lengeler et al., 1999). Methylated aromatic hydrocarbons (e.g., toluene and xylenes) are initially attacked by the enzyme, benzylsuccinate synthase (BssA), which adds fumarate (as above) to each molecule. After beta oxidation steps, the metabolic pathways leads to the key intermediate, benzoyl-CoA, shown in Figure 7.12. The current weight of evidence indicates that unsubstituted aromatic hydrocarbons (e.g., benzene, naphthalene) are initially carboxylated (to benzoic acid and naphthoic acid, respectively), before undergoing CoA ligation to benzoyl-CoA or naphthyl-CoA, respectively (Figure 7.12). These in turn undergo ring-reduction, hydratase, dehydrogenase, and ring-cleavage reactions (Safinowski and Meckenstock, 2006; Fuchs et al., 2011; Abu Laben et al., 2010; Meckenstock and Mouttaki, 2011; Heider, 2007) leading to central metabolism (especially via acetyl-CoA).

Oxidation states of elements predict compound reactivity

Biosphere compounds (e.g., Table 7.4), particularly those of carbon, sulfur, and nitrogen, exhibit a wide range of oxidation states. The importance of an elemental oxidation state, as a predictor of thermodynamic reactivity, was previously emphasized in Chapter 3 (Sections 3.6 to 3.9) and computation of elemental oxidation states was described in Box 3.4. Table 7.5 presents information that systematically declares the range of oxidation states of some of the carbon, nitrogen, and sulfur compounds listed in Table 7.4. The oxidation–reduction scale in Table 7.5 reminds the reader that the most oxidized forms of the elements (C as CO_2, S as SO_4^{2-}, and N as NO_3^-) are excellent electron acceptors for ATP generation in electron transport and other processes carried out by anaerobic microorganisms. At the opposite extreme, the most reduced forms of the elements (C as CH_4, S as H_2S, and N as NH_3) are excellent electron donors. Mid-range on the redox scale of Table 7.5 are compounds that can be oxidized *or* reduced. Examples of compounds exhibiting bidirectional (up and down) changes in oxidation states include: (i) carbohydrate (sugar) fermentation, in which a portion of the substrate pool is oxidized to CO_2 while the remainder of the pool is reduced to organic alcohols or acids (see Sections 3.8 and 3.10 and Figure 3.12); (ii) the disproportionation of thiosulfate by some anaerobic bacteria – generating

Table 7.5

Average oxidation states of carbon, nitrogen, and sulfur atoms in compounds found widely in soils, sediments, waters, and the atmosphere

	Carbon		Nitrogen		Sulfur	
Oxidation–reduction scale	**Compound**	**Oxidation state**	**Compound**	**Oxidation state**	**Compound**	**Oxidation state**
Oxidized (good electron acceptor)	CO_2 (carbon dioxide)	+4	NO_3^- (nitrate)	+5	SO_4^{2-} (sulfate)	+6
↓		0	NO_2^- (nitrite)	+3	$S_2O_3^{2-}$ (thiosulfate)	+2
			NO (nitric oxide)	+2		
			N_2O (nitrous oxide)	+1		
	CH_2O (carbohydrate, biomass tissue)		N_2 (dinitrogen gas)	0	$(CH_3)_2SO$ (dimethyl sulfoxide)	0
	$C_2H_4O_2$ (acetic acid)	0			S^0 (elemental sulfur)	0
Fermentable (disproportionation type reactions)	$C_3H_6O_2$ (propionic acid)	−0.66			$(CH_3)_2S_2$ (dimethyl sulfide)	−2
	$C_4H_8O_2$ (butyric acid)	−1				
	C_2H_6O (ethanol)	−2				
	C_8H_{18} (octane)	−2.25			$HSCH_2CHNH2COOH$ (cysteine)	−2
↓	CH_4 (methane)	−4	NH_3 (ammonia)	−3	H_2S (hydrogen sulfide)	−2
Reduced (good electron donor)						

both H_2S and SO_4^{2-}; and (iii) nitrite oxidation during the second major step of nitrification to nitrate, or nitrite reduction to NO in denitrification.

The cyclic nature of compound generation and destruction is apparent if information in columns 4 and 5 of Table 7.4 is scrutinized. To illustrate this key concept, the reader's attention should be focused on the elemental sulfur (S^0) entries in Table 7.4. Elemental sulfur is microbiologically produced via partial oxidation of the electron donor, sulfide, by chemolithoautotrophs. Remarkably, elemental sulfur has three potential metabolic pathways of destruction: (i) continued oxidation to sulfate by chemolithoautotrophs using S^0 as an electron donor and oxidants like O_2 as electron acceptors; (ii) reduction to sulfide in a process known as "sulfur respiration" by anaerobic *Archaea* and/or heterotrophic bacteria using S^0 as an electron acceptor; and (iii) oxidation of S^0 to sulfate by phototrophic sulfur bacteria requiring reducing power to fix CO_2.

Figure 7.13 reinforces the major theme begun in Chapter 3; thermodynamics and specific geochemical conditions set the stage for selective pressures that

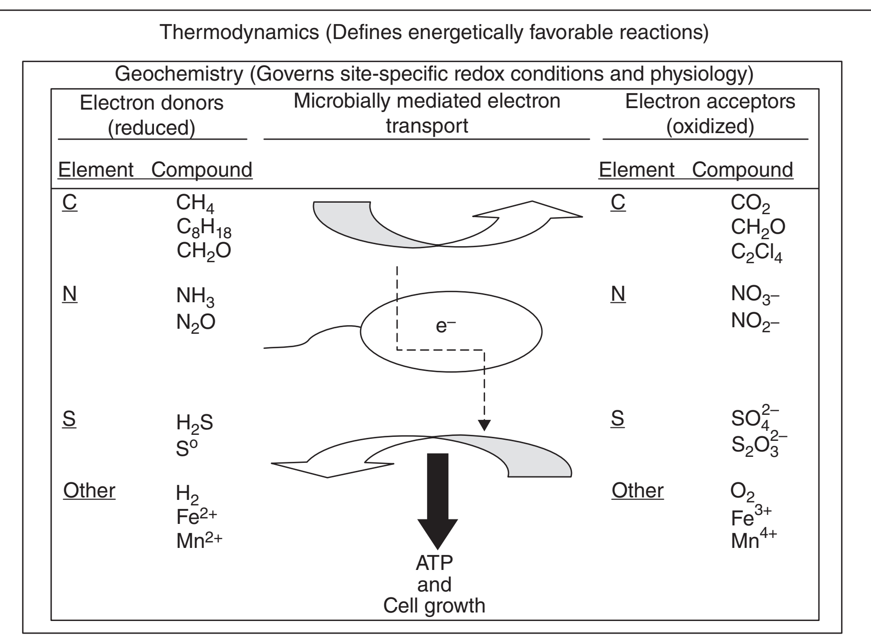


Figure 7.13 Conceptual view of how microorganisms catalyze biogeochemical reactions between electron donors and electron acceptors that occur in habitats such as soil, sediments, and waters. The specific reactions that occur in a given habitat are governed by both local geochemistry and the laws of thermodynamics. Each electron donor "half reaction" (upper curved arrow, center) must be coupled to an electron acceptor "half reaction" (lower curved arrow, center). (From Madsen, E.L. 2011. Microorganisms and their roles in fundamental biogeochemical cycles. *Cur. Opin. Biotechnol.* **22**:456–464. With permission from Elsevier.)

govern microbial evolution and physiology. ATP-generating electron transport in cells links reduced electron donors (C, N, S, and others) to oxidized electron acceptors. The main lesson that emerges from information in Table 7.4 and Figure 7.13 is that all biogeochemically produced substances cycle from one form to another. However, the multiple processes influencing the metabolic fate of any given biosphere compound are separated in space and time. Furthermore, *the metabolic process that prevails in any given ecological context depends upon the native microbial populations, the dominant terminal electron-accepting process (see Sections 3.7 and 3.8), ambient nutrient limitations, and ambient geochemical conditions*.

In the next few sections we will tackle biogeochemical cycles of individual elements. Our goal is to use the logic of physiology presented in Chapter 3 (Sections 3.6 to 3.11) to establish a rigorous foundation for systematically interpreting the catalog of biogeochemical reactions presented in Table 7.4.

7.4 ELEMENTAL BIOGEOCHEMICAL CYCLES: CONCEPTS AND PHYSIOLOGICAL PROCESSES

Complex mixtures of elements are cycled through food chains in nature

Chemically pure elements (O as O_2 gas, N as N_2, S as elemental sulfur, C as graphite, H as H_2 gas, Fe as the metal, etc.) can be found in the biosphere. However, in real-world habitats, such as soils, sediments, waters, and geologic formations, it is relatively rare to encounter any element in its pure state. Mostly, elements have a propensity to react with one another, either chemically or biochemically, thereby forming "compounds". As an example addressing inorganic compounds, O_2 (a strong oxidant) bonds readily with many elements – C, N, S, Fe, As, and H, creating, respectively, CO_2, NO_3^-, SO_4^{2-}, $Fe(OH)_3$, AsO_4^{3-}, and H_2O. Regarding organic compounds, the biomolecules that are the "stuff of life" are proteins, nucleic acids, carbohydrates, lipids, ATP, metalloenzymes – all of which are created through biosynthetic pathways that assemble compounds composed of the many nutrient elements.

At the level of ecological processes and trophic interactions (Figure 7.14), it is clear that the units of biomass transferred between primary, secondary, and tertiary consumers are the elementally heterogeneous tissues or entire bodies of creatures that dwell in the biosphere. Ecologically, it is important to note that two food chains operate in parallel in virtually every habitat. One food chain (left side, Figure 7.14) is based directly on net primary productivity (NPP; photosynthesis or chemolithoautotrophy). The other (right side, Figure 7.14) is based on the detritus food chain in which deceased biomass and fecal materials from the extant creatures are transformed into new microbial (decomposer) biomass, which, itself, is the base of a food chain that includes protozoa, nematodes, microarthropods, and other small animals.

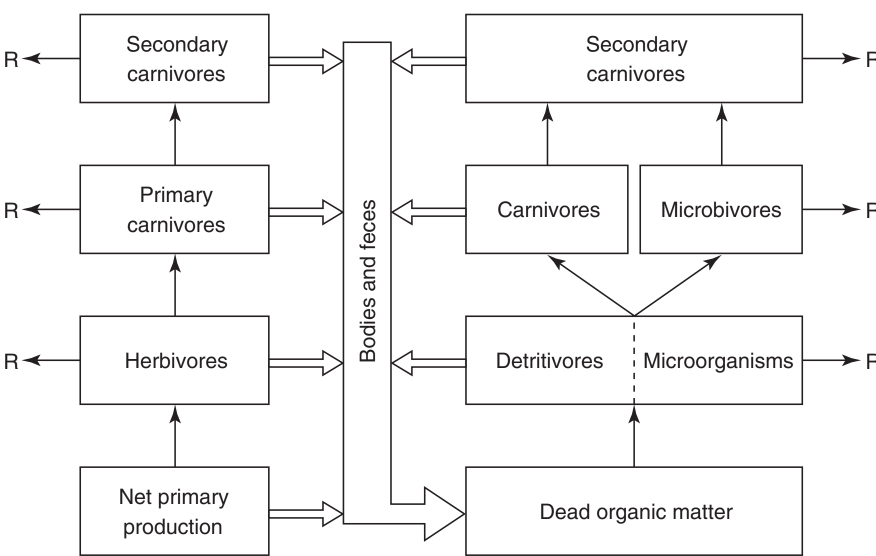


Figure 7.14 Trophic levels of ecosystems through two parallel food chains: one based on primary production and the other on dead biomass (detritus). The thin arrows show the flow of energy up the food chain (through living biomass) and the broad arrows show the complementary flow of detritus. R, respiration. (Reprinted from Staley, J.T. and G.H. Orians. 2000. Evolution and the biosphere. In: M.C. Jacobson, R.J. Charlson, H. Rodhe, and G.H. Orians (eds), *Earth System Science: From Biogeochemical Cycles to Global Change*, pp. 29–61. Academic Press, San Diego, CA. Copyright 2000, with permission from Elsevier.)

Thus, the real world is complicated and integrated. You cannot go out into a forest or lake or ocean or desert and find the isolated cycling of major elements (C, S, N, etc.; Canfield et al., 2010b). Nonetheless, the notion of discrete cycles of the elements is a useful exercise in biogeochemistry (see below).

Cyclic physiological processes: carbon, sulfur, and nitrogen

Thermodynamics (see Sections 3.6 to 3.8) gives us predictive power about microbially mediated reactions. Furthermore, the partial catalog of biosphere compounds (see Table 7.4 and Section 7.3) provides a glimpse of the pools of materials and their sources and sinks. It is conceptually insightful to split elements in real-world compounds away from one another. The exercise allows us to systematically assemble, focus, and integrate information describing how, when, and why biogeochemical processes occur.

Carbon cycle

Figure 7.15 provides a generic overview of how carbon cycles in the biosphere and the responsible physiological processes. The division in the

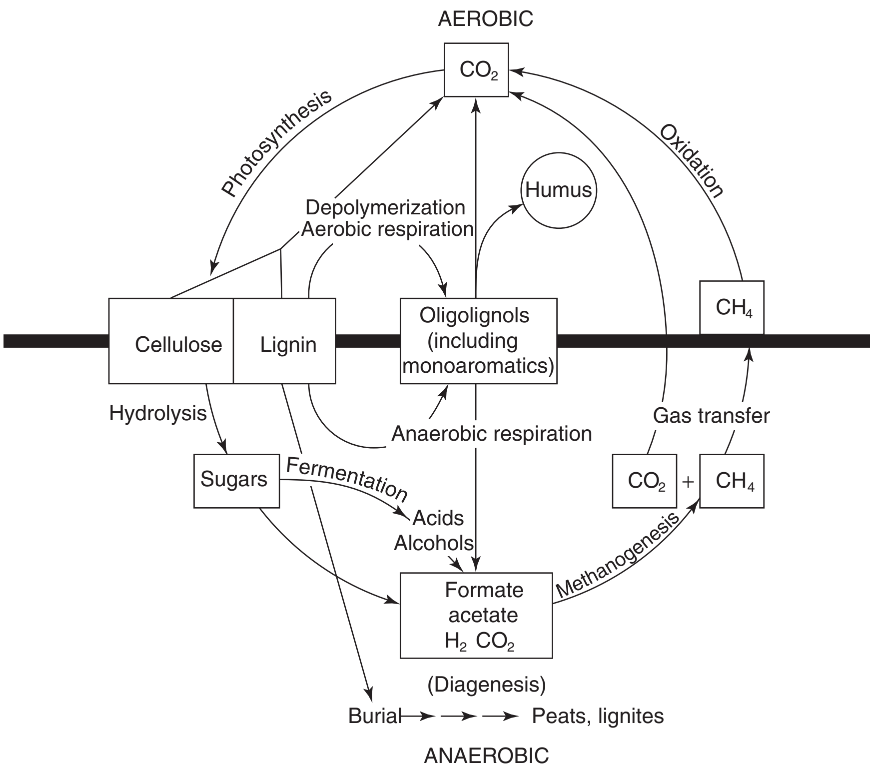


Figure 7.15 The carbon cycle, with emphasis on the processing of photosynthetically derived cellulose and lignin in aerobic and anaerobic habitats. (From Colberg, P.J. 1988. Anaerobic microbial degradation of cellulose, lignin, oligolignols, and monoaromatic lignin derivatives. In: A.J.B. Zehnder (ed.), *Biology of Anaerobic Microorganisms*, pp. 333–372. John Wiley and Sons, Inc., New York. Reprinted with permission from John Wiley and Sons, Inc., New York.)

diagram between aerobic (top half) and anaerobic (bottom half) processes reminds us of two important factors: (i) physiological conditions change in time and space and (ii) those changes have major implications for predicting thermodynamic instabilities – hence the processes that microorganisms can and do catalyze. Let us begin with CH_4 (right side, Figure 7.15). As mentioned in Table 7.4, methane is a thermodynamically stable endproduct of methanogenesis (dissimilatory reduction of CO_2 or fermentation of acetate or of methyl-containing compounds) in anaerobic habitats lacking non-CO_2 electron acceptors, but methane is the most reduced form of carbon (see Table 7.5) and serves as an electron donor for methanotrophic microorganisms that link electron flow generated during methane oxidation to the reduction of O_2 (Mancinelli, 1995; Murrell, 2010), NO_2^- (Raghoebarsing et al., 2006; Ettwig et al., 2010), SO_4^{2-} (Strous and Jetten, 2004; Milucka et al., 2012) and both Fe^{3+} and Mn^{4+} (Beal et al., 2009). These oxidations of CH_4 generate CO_2 (oxidation state of +4, Table 7.5),

which is a carbon source for autotrophs. Although there are five distinctive biochemical pathways for autotrophic CO_2 fixation (Thauer, 2007), Calvin cycle-carrying autotrophs (photosynthetic and chemosynthetic) predominate in biosphere habitats. Furthermore, *ribulose bisphosphate carboxylase* (RuBisCO) is the Calvin cycle's key enzyme. As indicated by the enzyme's title, ribulose bisphosphate carboxylase adds a CO_2 molecule to a phosphorylated form of the five-carbon sugar, ribose. This critical enzymatic step creates a six-carbon molecule, which is cleaved to two molecules of the three-carbon compound, 3-phosphoglycerate. Three turns of the Calvin cycle synthesizes the equivalent of one new 3-phosphoglycerate molecule – an essential biosynthetic building block that can lead to glucose, starch, and cellulose in plants and to gluconeogenesis in prokaryotes (via reversal of the glycolysis pathway; see Box 3.8).

Fixed CO_2 in plant or microbial biomass (shown as cellulose and lignin in Figure 7.15) has an intermediate oxidation state of ~0 (Table 7.5). Thus, biomass is susceptible to two potential physiological reaction pathways: (i) oxidation back to CO_2 by microorganisms using various electron acceptors (e.g., O_2, Fe^{3+}, Mn^{4+}, SO_4^{2-}) or (ii) reduction (often via fermentation) to an anaerobic intermediary metabolite pool of organic acids. The final step in Figure 7.15 completes the cycle by bringing us back to methane. As mentioned above, predominant mechanisms of methanogenesis use either H_2 as an electron donor and CO_2 as the acceptor creating methane or ferment (disproportionate) methyl-containing or C_1 compounds (e.g., formate, carbon monoxide, acetate, methanol, methylamine, or dimethylsulfide) to CH_4 and CO_2. Acetogenesis (discussed in Section 3.8) may also occur. Information in Box 7.4 illustrates a direct relationship between sources and sinks of methane and the atmospheric concentration of this greenhouse gas.

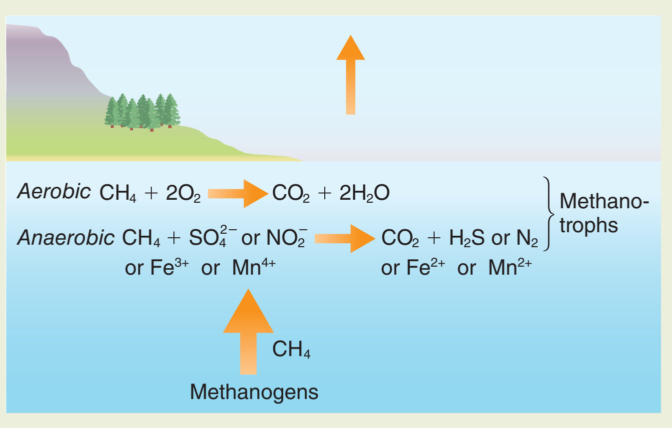
Sulfur cycle

A generalized view of the physiological sulfur cycle appears in Figure 7.16. As shown in Table 7.5, the fully reduced form of sulfur is hydrogen sulfide (H_2S; −2 oxidation state (–II); oxidation states are depicted in roman numerals on the left-hand axis of Figure 7.16). Fully reduced sulfur occurs in a variety of compounds – including inorganic sulfate minerals and organic compounds such as amino acids (Table 7.4) and *dimethyl sulfide* (DMS). Box 7.5 elaborates on the properties and environmental role of DMS in marine systems. At the opposite extreme, fully oxidized sulfur exhibits a +6 (+VI) oxidation state as the sulfate anion (top of Figure 7.16). Other forms of sulfur exhibit intermediate oxidation states, such as elemental S (zero oxidation state; see also Table 7.4). In the presence of oxidizing agents (for example, O_2 and nitrate), reduced forms of sulfur, especially H_2S and S^0 are thermodynamically unstable – they are used as electron donors for ATP production by chemolithotrophic microorganisms (see Section 3.11).

Box 7.4

Microbial controls on fluxes of methane to the atmosphere

In Section 7.2 it was pointed out that small changes in atmospheric methane concentrations have substantial impacts on global warming and climate change. There is a flux of methane from methane reservoirs and anaerobic sites of methanogenesis, which include soils, sediments, and the digestive tracts of termites, cows, and other ruminant animals. As methane diffuses up and away from its site of production (where it is a stable metabolic waste product), it migrates toward the atmosphere. In so doing, the methane passes through more oxidized zones where it is thermodynamically unstable – enriching methanotrophs that use SO_4^{2-}, NO_2^-, Fe^{3+}, and O_2 as final electron acceptors. In these neighboring habitats, methane becomes a useful physiological electron donor. Thus, the flux of methane that reaches the atmosphere is regulated (and substantially diminished) by methanotrophic microbial populations (see Sections 3.11, 7.3 and 7.5, and Boxes 7.9 and 7.10).



Lesson

Net methane flux to the atmosphere represents a balance between rates of production versus destruction. Clearly, understanding the microbial biogeochemical controls on these relative rates is of major scientific and biogeochemical concern.

Anaerobic, sulfur-rich surface waters, such as those in hot springs and Solar Lake in the Sinai Peninsula, harbor and release S^0 and H_2S. In these sites, bathed daily in sunlight, various groups of phototrophic sulfur bacteria utilize H_2S (an analog to the H_2O used in oxygenic photosynthesis) and S^0 as sources of electrons in photosynthetic dark reactions that fix CO_2, via RuBisCO-type reactions (see the above description of the C cycle). Arrows appear in Figure 7.16 representing this anaerobic phototrophic oxidation of reduced sulfur compounds (from H_2S to SO_4^{2-}, from H_2S to S^0, and from S^0 to SO_4^{2-})

After the various oxidation reactions have generated the sulfate anion, this material is stable in the presence of oxygen (e.g., most oceanic waters) but in carbon-rich sediments where microbial metabolic demand for electron acceptors (especially, O_2, NO_3^-, Fe^{3+}, and Mn^{4+}) exceeds supply, sulfate is an excellent electron acceptor. Sulfate-reducing prokaryotes convert sulfate to H_2S (a dissimilatory waste product); thus completing the physiological sulfur cycle.

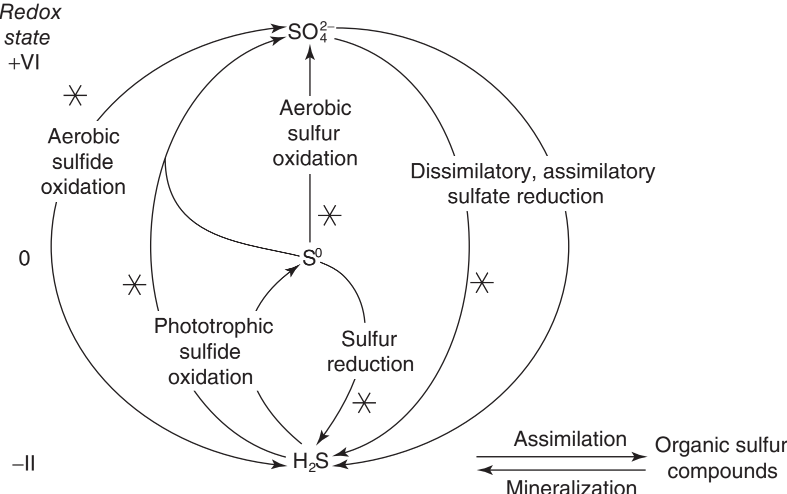


Figure 7.16 The sulfur cycle, showing key reactions and sulfur species. Note that the redox state of each sulfur compound is shown on the left axis in roman numerals. Reactions catalyzed exclusively by prokaryotes are marked with an asterisk. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 32.3. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

Nitrogen cycle

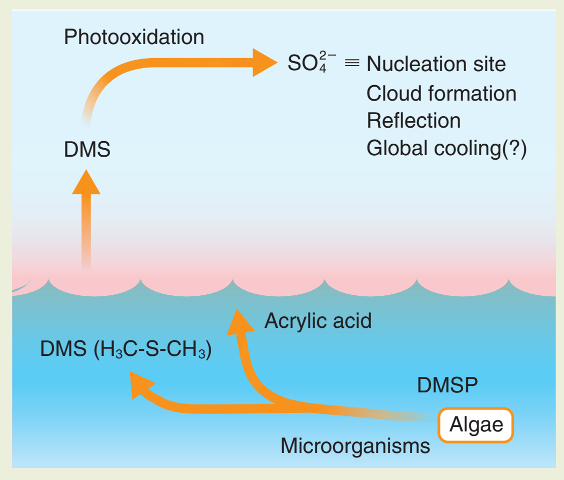
An overview of the physiological basis for nitrogen cycling is shown in Figure 7.17. The intricacies of microbial nitrogen transformations are suggested by the variety of nitrogen compounds and their respective oxidation states, shown in Tables 7.4 and 7.5. The basic principles used to organize reactions in the carbon and sulfur cycles apply also to nitrogen: the oxidized forms are useful final electron acceptors and the reduced forms are useful electron donors. Furthermore, a small portion of the vast atmospheric pool of N_2 (N≡N; zero oxidation state) can be brought into the biologically useful nitrogen pool via the uniquely prokaryotic process of nitrogen fixation, which creates intracellular NH_3 (−3 oxidation state). For a description of N_2 fixation, see Box 7.6.

Focusing on the upper left-hand portion of Figure 7.17, N_2 gas has been reduced to the NH_3 state (e.g., as amino acids) within a microbial cell represented as particulate organic nitrogen (PON). Cell lysis, hydrolysis of protein, and deamination of amino acids by heterotrophs (a three-step process known as *ammonification*) release the N as NH_4^+. PON can also be transported to anoxic waters where heterotrophic activity may convert particulate organic materials to the dissolved form (dissolved organic nitrogen, DON) and eventually to NH_4^+ (Figure 7.17).

In the oxic upper layers of ocean water NH_4^+ is thermodynamically unstable – it is used as an electron donor by chemolithoautotrophs in the

Box 7.5

Dimethyl sulfide: an unusual sulfur compound whose oceanic sources and transformations influence climate change



Marine algae (e.g., cocco lithophores and dinoflagellates) produce dimethyl sulfoniopropionate (DMSP) to maintain cellular osmotic balance in seawater. During decomposition of deceased algae, DMSP is used as a carbon and energy source by heterotrophic bacteria. The enzyme, DMSP lyase, splits the substrate into acrylate (related to propionate, a useful carbon and energy source) and dimethyl sulfide (DMS) – a volatile gas. As many as 50 million tons of DMS escape from oceans globally each year to the atmosphere where DMS can be oxidized by hydroxyl radicals to sulfate. Air-borne sulfate aerosol particles (originating from DMS) over oceanic waters are thought to contribute to cloud formation, whose reflectance may contribute to global cooling (Schlesinger, 1997; Yock, 2002; Vallina and Simo, 2007 Curson et al., 2011; Johnston et al., 2012).

Recent studies have made it clear that the global cooling effect of DMS is regulated by two additional marine microbial processes. The DMSP precursor for DMS can be assimilated directly from seawater by phytoplankton (both prokaryotic *Prochlorococcus* and eukaryotic algae such as diatoms; Vila-Costa et al., 2006). Also, widespread marine prokaryotic heterotrophs (such as *Silicibacter*, *Marinobacter*, and *Pelagibacter ubique*) carry genes that encode a demethylase enzyme that diverts DMSP down a metabolic pathway that eliminates DMS as a possible endproduct (Howard et al., 2006; Johnston et al., 2012; Curson et al., 2011). Both of the above processes may diminish the size and flux of the DMS pool in the oceans.

When DMS is produced (as above), it can also be transported to anaerobic habitats. There are three likely anaerobic metabolic fates of DMS: (i) during methanogenesis it may be converted to CH_4 and H_2S; (ii) as an electron donor for photosynthetic purple bacteria, DMS can be oxidized to dimethyl sulfoxide (DMSO); and (iii) a variety of chemoorganotrophs and chemolithotrophs oxidize DMS to DMSO to generate ATP using sulfate or nitrate as electron acceptors.

Clearly, the biogeochemistry of DMS represents an intricate balance between biological and chemical processes involving production, consumption, and transport. The genetic, genomic, biochemical, and evolutionary details of DMS continue to be advanced (Todd et al., 2007; Johnston et al., 2012; Curson et al., 2011).

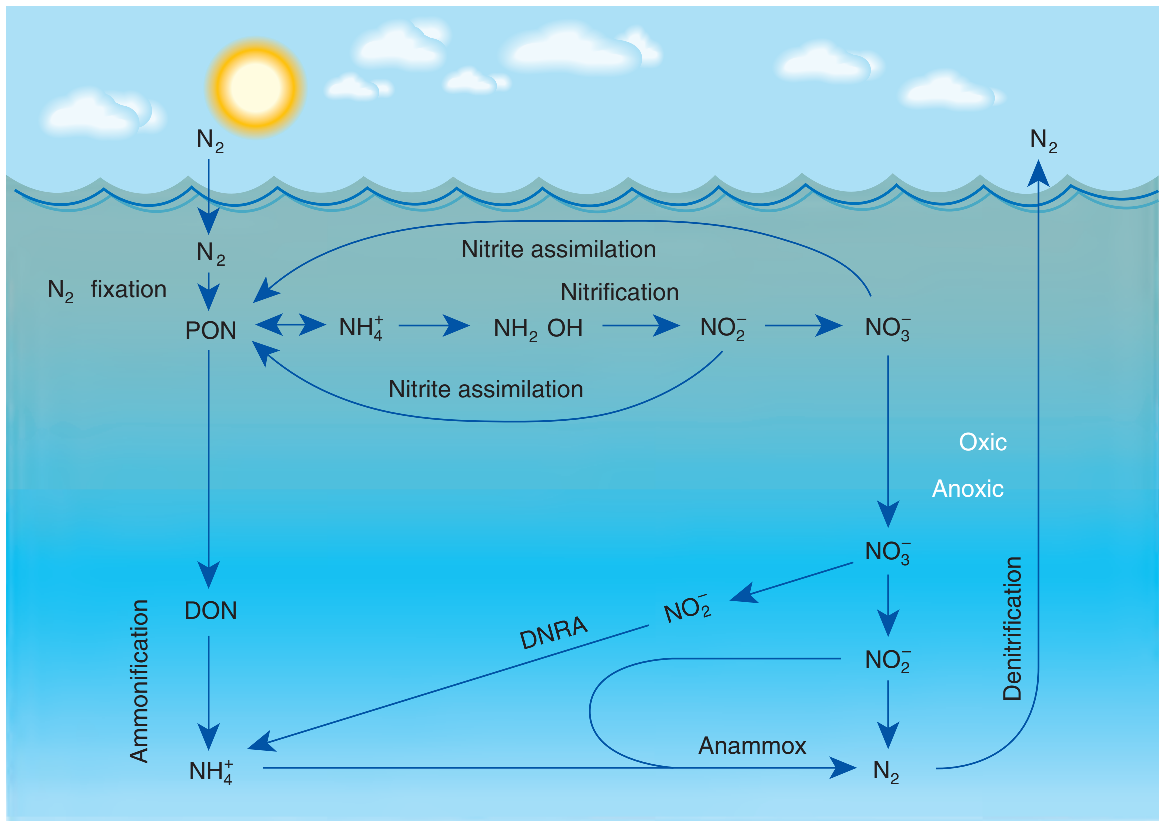


Figure 7.17 The nitrogen cycle. See text for details. DNRA, dissimilatory nitrate reduction to ammonium; DON, dissolved organic nitrogen; PON, particulate organic nitrogen, including phytoplankton. (Reprinted by permission from Macmillan Publishers Ltd: Nature, from Arrigo, K.R. 2005. Marine microorganisms and global nutrient cycles. *Nature* **437**:349–355. Copyright 2005.)

Box 7.6

Nitrogen fixation: an amazing and uniquely prokaryotic feat

Industrial and microbial processes fix N_2

The industrial source of commercial fertilizer nitrogen, the Haber–Bosch process, was invented in Germany around 1910. Using high pressure (200 atm or ~20,000 kPa) and temperature (450°C), atmospheric nitrogen (N_2; N≡N) is combined with H_2 gas to create NH_3. The prokaryotic enzyme complex, termed *nitrogenase*, carries out the same process at ambient temperatures and pressures.

Biology and biochemistry of nitrogen fixation

The overall reaction for biological nitrogen fixation is:

$$8H^+ + 8\,e^- + N_2 \rightarrow 2NH_3 + H_2$$

requiring an input of ~16 to 24 ATP.

The enzyme system also fortuitously acts on the triple bonded molecule acetylene HC≡CH, creating ethene $H_2C{=}CH_2$.

Two distinctive nitrogenase enzyme systems have been described in prokaryotes.

1 The most commonly distributed system (in free-living aerobic and anaerobic prokaryotes, some associated as symbionts with root structures in plants) delivers ATP and reducing power (as a reduced flavodoxin protein) to a multistep electron transport chain that ends with an Fe-Mo protein (dinitrogenase) that converts a single molecule of N_2 to two molecules of NH_3. Hydrogen gas is also produced (Figure 1). At least two additional structural variants in the dinitrogenase enzyme have been characterized – one with an Fe-V metal cluster and another with only Fe.

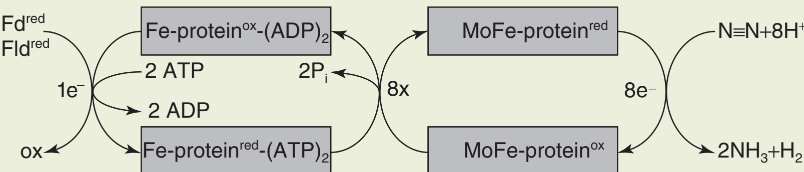


Figure 1 Model of the nitrogenase enzyme complex showing the transfer of electrons between protein carriers, ending in the release of ammonia and hydrogen. Fd, ferredoxin; Fld, flavodoxin. See box text for details. (From Lengeler, J.W., G. Drews, and H.G. Schlegel. 1999. *Biology of Prokaryotes*, fig. 8.14. Blackwell Science, Stuttgart. With permission from Blackwell Science, Stuttgart.)

The reaction is poisoned by molecular oxygen and aerobic prokaryotes (e.g., *Cyanobacteria*, *Azotobacter*, *Rhizobium*) have evolved clever and effective ways to shield their nitrogenase systems from atmospheric oxygen.

2 Another enzymatic strategy for nitrogen fixation has been recently described in *Streptomyces thermoautotrophicus* (Ribbe et al., 1997). Based on phylogenetic analysis of component proteins, this system appears to be an evolutionary hybrid – created by merging enzymes from the above (classic) nitrogenase system with other enzymes derived from the superoxide–dismutase oxygen-detoxification system. Remarkably, molecular oxygen is a required reactant in the electron-transport chain that delivers reducing power to a molybdenum-containing dinitrogenase enzyme.

multistep process known as *nitrification*. The microorganisms responsible for nitrification use O_2 as the final electron acceptor in their respiratory chains to generate a proton motive force and ATP. Some populations (e.g., *Nitrosomonas* sp.) oxidize NH_4^+ to NO_2^-. Others (e.g., *Nitrobacter* sp.) complete the process by oxidizing NO_2^- to NO_3^-. For decades, only *Bacteria* were known to carry out ammonia oxidation; isolated cultures (such as *Nitrosomonas*) were the sole sources of biochemical, genetic, and genomic insights about nitrification. In recent years, *Archaea* have been recognized to be key players in nitrification-related processes in soil and in marine systems (Stahl and de la Torre, 2012; Santoro et al., 2011; Agogué et al., 2008). Additional details of nitrification and denitrification are provided in Box 7.7.

Box 7.7

Ways that denitrification and nitrification influence greenhouse gases and climate change

Section 7.2 presented the crucial roles that N_2O and ozone play in tropospheric global climate change. Surveys of gas fluxes into the atmosphere from terrestrial soils have proven that two nitrogen-containing gases, N_2O (nitric oxide) and NO (nitrous oxide), are routinely released. A sample data set from Amazon basin soils is shown in Table 1.

Table 1

Summary of annual N_2O and NO soil emissions from the Amazon basin (from Neill, C., P.A. Steudler, D.C. Garcia-Montiel et al., 2005. Rates and controls of nitrous oxide and nitric oxide emissions following conversion of forest to pasture in Rondonia. *Nutr. Cycling Agroecosyst*. **71**:1–15, table 2. With kind permission of Springer Science and Business Media)

	N_2O (kg N/ha/year)		NO (kg N/ha/year)	
Location	**Forests**	**Pastures**	**Forests**	**Pastures**
Nova Vida, Rondonia	1.7–2.0	3.1–5.1		
	1.2–1.7			
Nova Vida, Rondonia	4.3	0–1.3	1.4	0.1–0.4
Noval Rondonia	3.2			
Nossa Senhora, Rondonia				0.17
Paragominas, Parú	2.4	1.5	1.5	1.0
		0.2		0.6
Manaus, Amazonas	1.4			
Manaus, Amazonas	1.9	6		
Tapajos, Pará	2.3		1.7	

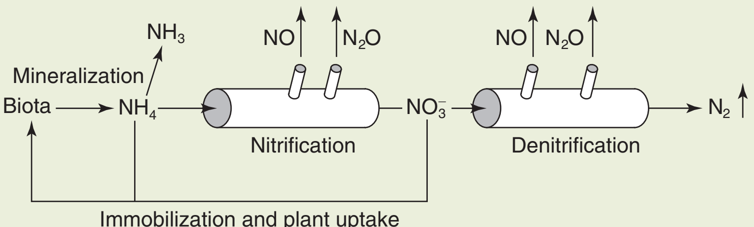


Figure 1 Microbial processes that yield nitrogen gases during nitrification and denitrification in soil. (From Schlesinger, W.H. 1997. *Biogeochemistry: An Analysis of Global Change*, 2nd edn. Academic Press, San Diego, CA. Copyright 1997, with permission from Elsevier.)

It has been documented that both nitrification and denitrification biochemical pathways leak both NO and N_2O to the atmosphere. A broad scheme depicting N processing in terrestrial systems is shown in Figure 1. Special emphasis is given to gaseous release of NO and N_2O.

Biochemical basis of gas production

According to Conrad (Remde and Conrad, 1991; Conrad, 1996), production of NO and N_2O from soil are a composite of several different processes. In denitrification, NO and N_2O are directly produced during stepwise reduction of nitrate to N_2 (Figure 2). However, intracellular retention of NO and N_2O is not 100% efficient: a portion of these gases escape from the metabolic pathways and reach the atmosphere (Davidson et al., 2000, 2004).

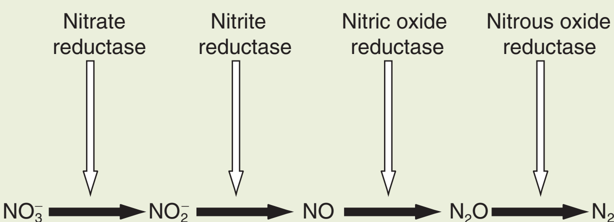


Figure 2 Summary of denitrification: outline of the pathway and enzymes involved. (Reprinted from Wrage, N., G.L. Velthof, M.L. van Beusichem, and O. Oenema. 2001. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biochem*. **33**:1723–1732. Copyright 2001, with permission from Elsevier.)

In nitrification, N_2O is formed during chemical decomposition of two intermediary metabolites (NH_2OH and NO_2^-; Figure 3). In addition, within a single bacterium capable of both nitrification and denitrification, the intermediary nitrification metabolite, NO_2^-, can be intercepted by the denitrification enzyme, nitrite reductase, yielding NO. It is important to note that nitrite is also chemically unstable, especially at low pH – producing NO.

Box 7.7 ***Continued***

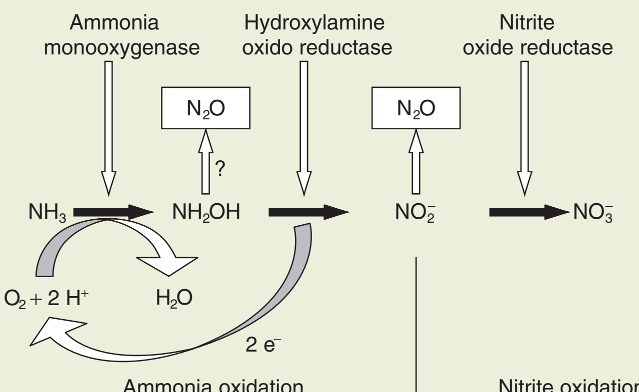


Figure 3 Summary of nitrification: outline of the pathway and enzymes involved. The white, upward-pointing arrows indicate chemical decomposition. (Reprinted from Wrage, N., G.L. Velthof, M.L. van Beusichem, and O. Oenema. 2001. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biochem.* **33**:1723–1732. Copyright 2001, with permission from Elsevier.)

Atmospheric effects of N_2O and NO

N_2O is a greenhouse gas that contributes to global warming in the lower atmosphere. Furthermore, in the upper atmosphere, N_2O decomposes to a reactive species that consumes the ozone layer that shields the biosphere from DNA-damaging UV radiation. NO is not directly a greenhouse gas, but its reactions in the lower atmosphere lead to the formation of ozone, a pollutant and a greenhouse gas.

In an aerobic habitat, NO_3^- is thermodynamically stable. However, when NO_3^- is transported to an anaerobic habitat it becomes an excellent final electron acceptor for anaerobes. As shown in the lower right region of Figure 7.17, there are three interacting processes that influence nitrate and its metabolites in anaerobic habitats: denitrification, dissimilatory nitrate reduction to ammonia (DNRA), and anammox.

1 In the (classic) *denitrification process*, single anaerobic microorganisms use NO_3^- as their terminal electron acceptor to fuel an ATP-generating respiratory chain. The intracellular endproduct is N_2 gas (produced via several intermediary metabolites: NO_2^- (nitrite, a key reactant for anammox), NO (nitric oxide), and N_2O (nitrous oxide); see Box 7.7). While the fully integrated, four-step denitrification process (nitrate reductase → nitrite reductase → nitric oxide reductase → nitrous oxide reductase; shown in Box 7.7) is a valuable paradigm, there are many

"exceptions to the rule" (Shapleigh, 2013). For example, the accumulating roster of completed microbial genomes has revealed that many organisms carry incomplete genetic denitrification pathways – implying the occurrence of truncated metabolism with the potential to release both NO and N_2O from cells. Also surprisingly, the first two steps of this pathway are often expressed under fully or partially aerobic conditions. Concern over release from soils of the powerful greenhouse gas, N_2O, demands tools for tracking the occurrence and expression of nitrous oxide reductase (encoded by *nosZ*); yet very recently it has been shown that tools for monitoring *nosZ* have been inadequate because of unexpected genetic diversity of *nosZ* in soil microbial populations (Sanford et al., 2012). Another key recent revelation affecting N_2O release from soil is the cellular stability of the enzyme, N_2O reductase: acidic conditions appear to interfere with enzyme assembly, leading to impaired N_2O-reductase activity, hence enhanced release of N_2O from cells (Bakken et al., 2012).

2 *Dissimilatory nitrate reduction to ammonia* (DNRA) has been well described in enteric bacteria (like *Escherichia coli*; these inhabit digestive tracts of animals). DNRA is thought to be a physiological strategy that allows cells to avoid an intracellular excess of reducing equivalents and/or reduced toxic metabolites. Note that reduction of nitrate to ammonia consumes eight electrons, while reduction of nitrate to N_2 consumes five electrons. Although thermodynamic calculations indicate a lower free-energy yield for DNRA versus denitrification, Strohm et al. (2007) showed that cellular growth yields were actually higher for DNRA. This unexpected discrepancy was attributed to inefficient energy conservation by the respiratory chain of denitrifiers.

- **Question:** In a given anaerobic habitat containing nitrate, what determines if the respiratory flow of electrons yields ammonia (DNRA) or N_2 (denitrification)?
- **Answer:** Current theory is that the answer lies in the ratio of electron donor (usually C) to electron acceptor (NO_3^-). DNRA is favored when C/NO_3^- ratios in a given locale are high, when the electron acceptor is limiting (see especially Kraft et al., 2014).

The extent that DNRA occurs in natural habitats (Figure 7.17; Tiedje, 1988; Welsh et al., 2001; Rütting et al., 2011; Kraft et al., 2014) is uncertain. ^{15}N-tracer techniques indicate that DNRA may be extensive in some soil habitats (Rütting et al., 2011); this may be highly significant, ecologically, because ammonia is retained by the ecosystem (not released as N_2) when DNRA occurs. In addition, Lam et al. (2009) have shown that DNRA plays a major role in N cycling in Pacific Ocean water off the coast of Peru.

3 *Anammox* stands for "anaerobic ammonia oxidation". In this reaction, fully reduced NH_4^+ is used as an electron donor by chemolithoautotrophs that utilize NO_2^- as the electron acceptor. The recently discovered

anammox reaction (see Section 7.5 and Box 7.8) is carried out by members of the *Planctomyces* phylum of *Bacteria* (especially *Candidatus* Kuenenia stuttgartiensis) that exhibit unique ("ladderane") membrane lipids that form an intracellular "anammoxosome" organelle – the site of membrane-bound enzymes that oxidize nitrite to nitric oxide (NO) and then combine NO with ammonia to create hydrazine (N_2H_4), which is oxidized to N_2 gas. Electron flow in this process generates ATP (Kuypers et al., 2003; Strous and Jetten, 2004; Strous et al., 2006; Kartal et al., 2011). Current estimates are that anammox may account globally for 30–50% of N_2 production in the ocean (Arrigo, 2005), though the proportion could actually be much higher (Kuypers et al., 2005). Indeed, the ecological significance of anammox has been confirmed by Lam et al. (2009), who quantified linkages between many N-cycling processes in the western South Pacific (see also Lam and Kuypers, 2011).

Box 7.7 describes the impacts of two key nitrogen cycle processes (denitrification and nitrification) on global warming.

Science and the citizen

Arsenic-contaminated drinking water and the role of biogeochemistry

Headline news from the World Health Organization (WHO): arsenic-contaminated drinking water threatens the lives and wellbeing of tens of millions of people worldwide, particularly in Bangladesh and West Bengal, India

Arsenic is a toxic element capable of existing in five different valence states (As (–III), (0), (II), (III), and (V)). Forms of arsenic range from sulfide minerals (e.g., As_2S_3) to elemental As to arsenic acid to arsenite (AsO_2^-) to arsenate (AsO_4^{3-}) to various organic forms that include methylated arsenates and trimethyl arsine. The dominant form of inorganic arsenic in aqueous, aerobic habitats is arsinate (oxidation state of +5 (V)). In contrast, arsenite (oxidation state of +3 (III)) occurs widely in anaerobic aqueous environments (Oremland and Stolz, 2003; Mukhopadyay et al., 2002).

Arsenic has been used by humans as a medicinal agent, a pigment, a pesticide, a poison, and in the production of glass and semiconductors. Inorganic forms of arsenic are more toxic than organic forms. In arsenite, the mobile trivalent (III) form is highly toxic and reacts with thiol groups of proteins within living cells; this rapidly disrupts many types of metabolism, particularly energy generation in the Krebs citric acid cycle. Though arsenate, the pentavalent (V) form of arsenic, exhibits a somewhat diminished toxicity and mobility, its resemblance to phosphate allows arsenate to interfere with respiratory processes – acting as an uncoupling agent in oxidative phosphorylation. Very few human organ systems escape the toxic effects of arsenic.

Arsenic is listed as a presumed carcinogenic substance based on the increased prevalence of lung and skin cancer observed in human populations with multiple exposures. Chronic

exposure through drinking water leads to a variety of characteristic skin lesions (Figure 1). Globally, an estimated 100 million people are at risk of exposure to unacceptable arsenic levels in drinking well-water supplies (Islam et al., 2004).

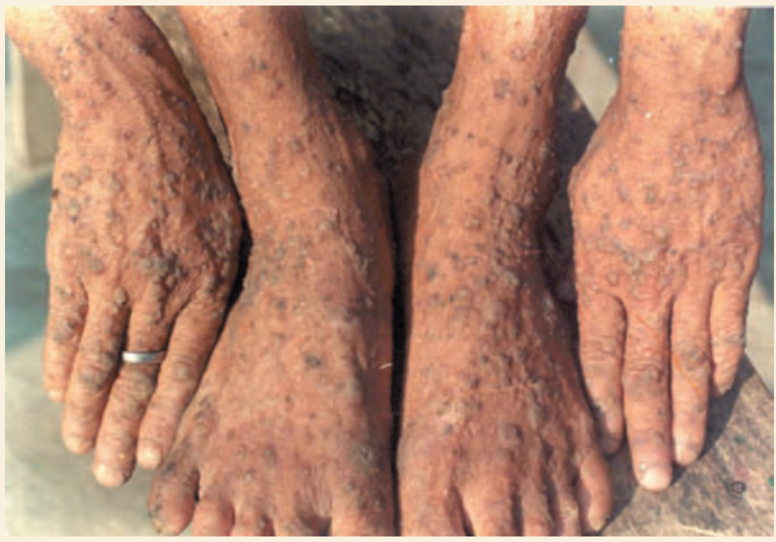


Figure 1 Subject from a village in West Bengal, India with the full panoply of arsenical skin lesions, including hyperkeratosis, suspected Bowen's disease, and nonhealing ulcers (suspected cancer). (Photo courtesy of Dr. Dipankar Chakraborti, Jadavpur University, India, with permission.)

SCIENCE: microbial processes that govern arsenic biogeochemistry

By studying Mono Lake in California, Oremland and Stolz (2003) have established the fundamental physiological and biogeochemical principles that apply to microbially mediated oxidation/reduction reactions of arsenic (Figure 2). The water column in Mono Lake, about 30 m deep, supports a gradient from oxic to anoxic conditions (left side, Figure 2). In a mid-gradient transition zone, arsenite (As(III)) is used as an electron donor by aerobic chemolithoautotrophic arsenite-oxidizing microorganisms (CAOs, center of Figure 2), producing arsenate (As(V)). Farther down in the water column in the absence of oxygen, heterotrophic microorganisms are limited by the availability of final electron acceptors. Fueled by reduced carbon (CH_2O) raining down from the upper water column, dissimilatory arsenate-reducing prokaryotes (DARPs, right side of Figure 2) use arsenate as an electron acceptor, thus completing the local arsenic redox cycle. An interesting variation on Mono Lake's As biogeochemistry has been described by Hollibaugh et al (2006): the microbial community can couple arsenate reduction to the oxidation of reduced S in the form of sulfides.

In Bangladesh and India, arsenic is thought to originate from naturally occurring minerals in the highlands of the source rivers (Chakraborti et al., 2003). Though the same

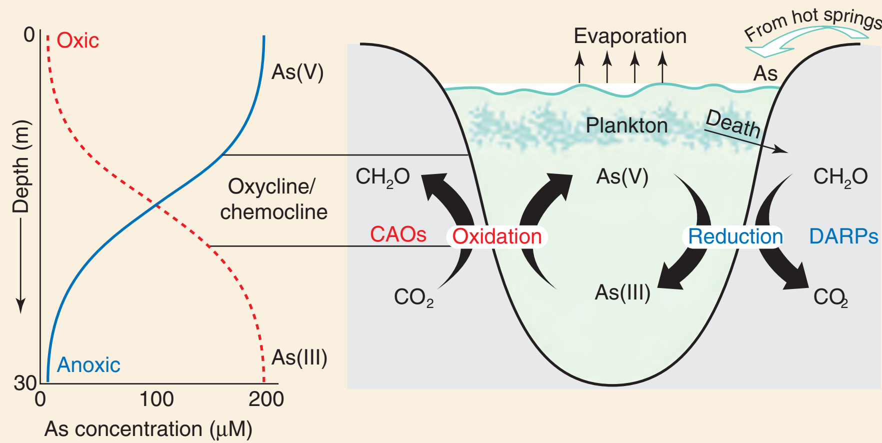


Figure 2 The chemical speciation of arsenic in the stratified water column of Mono Lake, California (left) as explained by the metabolism of arsenic by microbial populations present in the water column (right). Arsenic cycling occurs in the region of the chemocline. Arsenate reduction is mediated by DARPs that use released organic matter from dying plankton to fuel their respiration. Arsenite oxidation (aerobic and anaerobic) is mediated by CAOs that also contribute to secondary production by "fixing" CO_2 into organic matter. Arsenic first enters this alkaline (pH = 9.8), saline (290 g/l) lake as a dissolved component contained in the discharge from hydrothermal springs. Arsenic, as well as other dissolved constituents, reaches high concentrations because of the predominance of evaporation over precipitation in this arid region. (From Oremland R.S. and J.F. Stolz. 2003. The ecology of arsenic. *Science* **300**:939–944. Reprinted with permission from AAAS.)

microbiological principles of arsenic apply to both Mono Lake and Asia, the delta regions of India and Bangladesh are distinctive in several ways: (i) there is a high human population density; (ii) agricultural activities that include irrigation and the use of nitrate fertilizers; (iii) the installation of tube wells (Figure 3) for extracting drinking water; and (iv) the presence of iron minerals in the sediments. The iron minerals interact both chemically and physically with the arsenic. New FeAs minerals can form. Also, mineral surfaces act as sorption sites that can bind and influence the mobility of arsenate and arsenite. In addition, Fe(III) may be used in preference to arsenate as an electron acceptor by anaerobic microorganisms (Islam et al., 2004).

The overall biogeochemistry of the tube-well systems (Figure 3) is complex. Interactions between multiple electron donors (organic carbon, sulfide, arsenite, Fe(II)) and acceptors (oxygen, nitrate, arsenate, Fe(III)) govern microbial processes. In turn, the microbial processes are influenced by geochemical reactions. Moreover, the setting is dynamic in space and time – reflecting both climate-related hydrologic events and human-managed agricultural practices. There is a pressing need to understand and manage this biogeochemical system because of its impact on the wellbeing of millions of people.

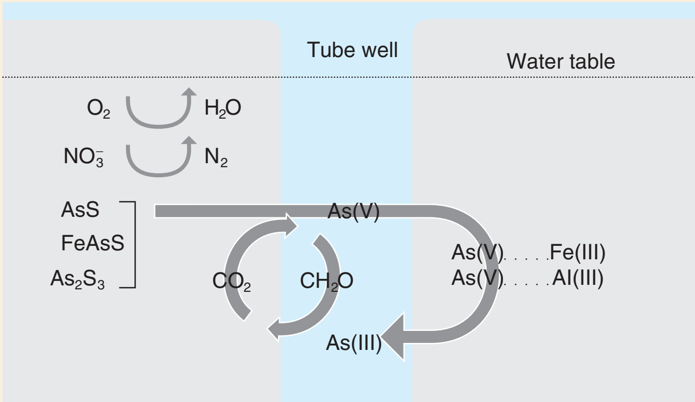


Figure 3 A conceptual model of how arsenic-metabolizing prokaryotes may contribute to the mobilization of arsenic from the solid phase into the aqueous phase in a subsurface drinking water aquifer. Arsenic is originally present primarily in the form of chemically reduced minerals, like realgar (AsS), orpiment (As_2S_3), and arsenopyrite (FeAsS). These minerals are attacked by chemolithoautotrophic arsenite-oxidizing microorganisms, which results in the oxidation of As(III), as well as iron and sulfide, with the concurrent fixation of CO_2 into organic matter. Construction of wells by human activity accelerates this process by providing the necessary oxidants like molecular oxygen or, in the case of agricultural regions, nitrate. The As(V) can subsequently be adsorbed onto oxidized mineral surfaces like ferrihydrite or alumina. The influx of substrate organic materials derived either from buried peat deposits, recharge of surface waters, or the microbial mats themselves promote microbial respiration and the onset of anoxia, and hence the conversion of As(V) to mobile As(III). DARPs then respire adsorbed As(V), resulting in the release of As(III) into the aqueous phase. (From Oremland R.S. and J.F. Stolz. 2003. The ecology of arsenic. *Science* **300**:939–944. Reprinted with permission from AAAS.)

Research essay assignment

Select an element other than carbon, sulfur, nitrogen, or arsenic and prepare an essay on its biogeochemistry. At the beginning of the essay, declare what motivated your selection of the element. Are you concerned with public health issues? Environmental pollution issues? Evolutionary issues? Or physiological or other issues?

Use the published literature (books, peer-reviewed publications) to develop a presentation that includes: (i) the element's biogeochemical cycle; (ii) key physiological steps of the cycle; and (iii) an application of the cycle to everyday life of people.

7.5 CELLULAR MECHANISMS OF MICROBIAL BIOGEOCHEMICAL PATHWAYS

Learning the biochemical and genetic mechanisms of infectious disease provides myriad opportunities for medicine-based disease prevention. Mastering the intricacies of biogeochemical reactions can also lead to

biotechnology developments (see Section 8.6) and to wise ecological management practices. In the classic approaches to discovering biochemical pathways (for instance, photosynthesis in algae – the Calvin cycle of CO_2 fixation), a model organism that carries out the biochemical process needs to be grown in large quantities in the laboratory. Then labeled substrates ("tracers", composed of radioactive atoms or rare stable isotopes) can be added to the active cells and the metabolic pathway can be discovered using analytical chemistry procedures to identify sequential metabolites. The structures of the metabolic intermediate compounds, themselves, are the basis for hypotheses about biochemical conversions and rates. Next, extracted enzymes from the dense cell preparations can be separated from one another and the individual enzyme activities can be tested and proven to carry out individual, sequential steps in the metabolic pathways. Furthermore, the genes encoding each enzyme can be identified in the test organisms (e.g., via site-directed mutagenesis (see Sections 6.9 and 9.2, and Table 6.4) or by determining the amino acid sequence of the protein, inferring the DNA template encoding the protein, and then probing genomic DNA extracts and sequencing the identified genes). Typically, next examined are intra- and extracellular signaling networks (regulatory proteins and their biochemical cues) that control and modulate expression of the genes that encode the processes of interest. Topics explored by structural biological investigations add to understanding by revealing the three-dimensional arrangement of atoms in enzymes and of enzymes in membranes.

The types of biochemical and genetic studies described above have been extended to each of the biogeochemical processes shown in Figures 7.15 to 7.17 and listed in Table 7.4. Consequently, there is an enormous amount of scientific information available about physiological, biochemical, and genetic mechanisms of microbially mediated reactions. As an example, Table 7.6 provides a listing of many microbial systems and their enzymes that catalyze nitrogen cycling.

The anatomy of discovery: anaerobic oxidation of ammonia and methane

As discussed in Chapter 6 (Sections 6.6 and 6.11), selecting an ecologically important model organism (or consortium of enriched populations) as a source of biochemical and genetic information is a challenge. That challenge was met exceptionally well in three recent and remarkably successful cases that each uncovered and characterized microbial processes of global significance: anaerobic ammonia oxidation (anammox, Box 7.8) and sulfate-based anaerobic methane oxidation (AMO, Box 7.9) and nitrite-based AMO (Box 7.10).

Table 7.6

Types of microorganisms active in the nitrogen cycle and their enzyme systems. (Modified from Stein, L.Y. and Yung, Y.L. 2003. Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide. *Annu. Rev. Earth Planet. Sci*. **31**:329–356. Reprinted with permission from *Annual Reviews of Earth and Planetary Sciences*, Vol. 31. Copyright 2003 by Annual Reviews, www.annualreviews.org)

Bacterial group	Process	Main enzyme	Metabolism
Chemolithotrophic ammonia oxidizers	NH_3 oxidation to NO_2^- via NH_2OH	AMO	Dissimilatory
	NH_2OH oxidation to NO and N_2O	HAO	
	NO_2^- reduction to NO	HAO or nonenzyme	?
	NO reduction to N_2O	NIR	?
	NO_2^- reduction to N_2	NOR	?
Methane oxidizers	NH_3 oxidation to NO_2^- via NH_2OH	?	?
	NH_2OH oxidation to NO and N_2O	MMO	Fortuitous*
	NO_3^- reduction to NO_2^-	P460	
	NO_2^- reduction to NH_3	P460 or nonenzymatic	?
	N_2 fixation to NH_3	?	Assimilatory
	NO_2^- reduction to NO	?	Assimilatory
	NO reduction to N_2O	Nitrogenase	Assimilatory
Nitrite-driven anaerobic methane oxidation	2NO to N_2 and O_2	NOD, nitric oxide dismutase	Dissimilatory
Heterotrophic ammonia oxidizers	NH_3 oxidation to NO_2^- via NH_2OH	NIR	?
Nitrite oxidizers	NO_2^- oxidation to NO_3^-	NOR	
	NO_3^- reduction to NO_2^- NH_3, and N_2O	AMO	Fortuitous
	NO oxidation to NO_2 and NO_3^-	HAO	
Dissimilatory denitrifiers	NO_3^- reduction to NO_2^-	Nitrite oxidase	Dissimilatory
	NO_2^- reduction to NO	Nitrite oxidase	
	NO reduction to N_2O	?	
	N_2O reduction to N_2	?	?
	NO oxidation to NO_3^-	NAR, NAP	Dissimilatory
	NO_3^- reduction to NO_3^- via NO_2^-	NIR	Dissimilatory
Assimilatory denitrifiers	NO_3^- reduction to NO_2^-	NOR	Dissimilatory
	NO_2^- reduction to NH_3	NOS	
Fungal denitrifiers	NO_2^- reduction to NO	N_2OR	Dissimilatory
	NO reduction to N_2O	?	?
Nitrogen fixers	N_2 to NH_3	NAR	Dissimilatory
Anammox	NH_3 and NO_2^- to N_2 via N_2H_4 and NH_2OH	NAS	
		NAS	Assimilatory
		Siroheme NIR	Assimilatory
		Hexaheme NIR	
		Cu-NIR	?
		P450nor	?
		Nitrogenase	Assimilatory
		Hydrazine synthase	Dissimilatory
		Hydrazine dehydrogenase	Dissimilatory

AMO, ammonium monooxygenase; Cu-NIR, copper-based nitrite reductase; HAO, hydroxlyamine oxidoreductase; MMO, methane monooxygenase; NAP, nitrate reductase (in periplasm of cell); NAR, nitrate reductase (membrane bound); NIR, nitrite reductase; NOR, nitric oxide reductase; NOD, nitric oxide dismutase; N_2OR, nitrous oxide reductase; NAS, nitrate assimilation; NOS, nitrous oxide reductase; P450nor, cytotochrome P450 nitric oxide reductase; P460, cytochrome P-460.

*Fortuitous here means of no direct physiological benefit; caused by nonspecific enzymes active in an unrelated process (co-metabolism).

Perusal of the information in Boxes 7.8, 7.9, and 7.10 reveals interesting commonalities and contrasts in the progression of experiments that established anaerobic oxidation of ammonia and methane within biogeochemistry. As noted by Strous and Jetten (2004), both ammonium and methane are biochemically difficult to attack. Their activation under aerobic conditions by monooxygenase enzymes makes use of oxygen's tendency to steal electrons via free-radical reactions. Notions about anaerobic metabolism of ammonium and methane originated with chemical oceanographers (Boxes 7.8 and 7.9). Physiological documentation of the processes and elucidation of biochemical reaction mechanisms followed decades later. Both the paths of discovery began more than 50 years ago and both were hampered by scientific dogma

Box 7.8

Timeline and events in the discovery of anaerobic ammonia oxidation (anammox): from a denitrifying bioreactor to global cycling of nitrogen

1. *1965–1977. Chemical oceanographers speculate*. Thermodynamic calculations and ratios of nutrients in both ocean waters and phytoplankton ("Redfield ratios") suggest anoxic metabolism of ammonia (Strous et al., 1999).
2. *1990. Experimental verification of physiological process in engineered bioreactor systems*. Mass balances and stoichiometric relationships between compounds entering and exiting flow-through vessels colonized by microorganisms show that nitrite and ammonia are simultaneously consumed (Strous and Jetten, 2004).
3. *1995. Laboratory growth conditions of an enrichment culture show nitrate and ammonia are essential reactants for microbial growth of enriched, transferable microorganisms*. Sequencing of 16S rRNA genes identify the dominant organisms as a member of the *Planctomyces* phylum (Strous et al., 2002; Strous and Jetten, 2004).
4. *1999–2002. Dominant organisms in enrichment culture are physically purified – allowing detailed physiological studies*. Intermediary metabolites are confirmed – especially one unique to biology, hydrazine (H_2NNH_2). Autotrophy (CO_2 fixation) is shown to be linked to NH_4 oxidation. Purification of key enzyme: hydroxylamine/hydrazine oxidoreductase is housed in a membrane-bound organelle (the anammoxosome) composed of unique ladderane lipids (Jetten et al., 2003; Strous and Jetten, 2004). (For a metabolic scheme, see Figure 1 in Box 7.9.)
5. *2003–2005. Widespread geographic significance (Black Sea and coasts of Africa and Costa Rica)*. In situ geochemical profiles show NH_4 depletion in nitrite-rich zones. Microbial communities incubated with $^{15}NH_4^+$ form N_2 with mixed isotopic composition (^{14}N ^{15}N), which distinguishes anammox from the denitrifying pathway of anaerobic N_2 formation (Kuypers et al., 2003, 2005) (Figure 1).

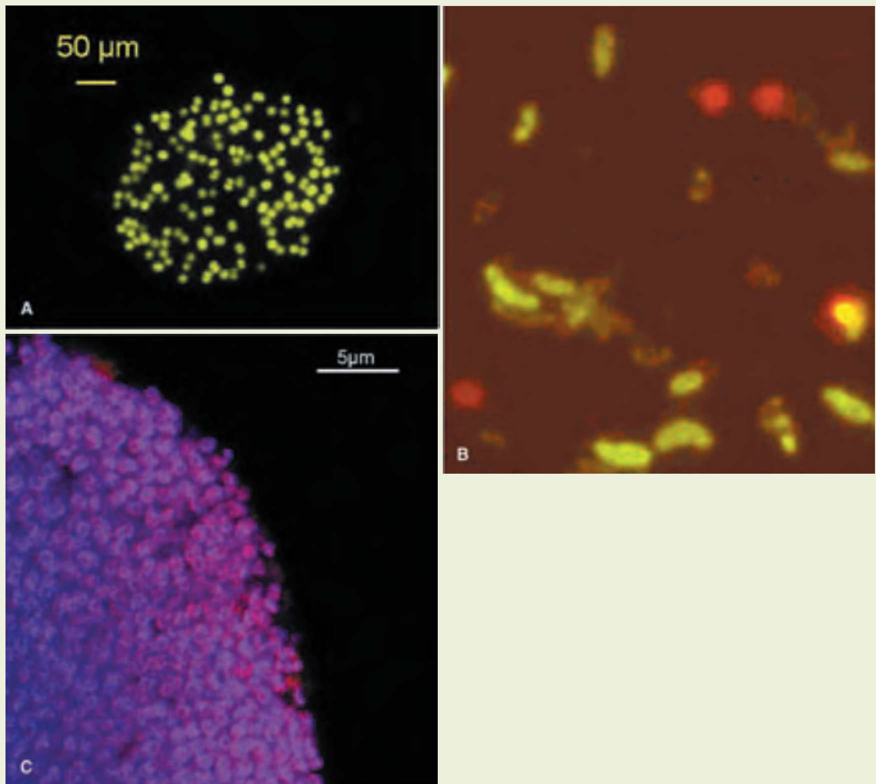


Figure 1 Microscopic images using fluorescent in situ hybridization (FISH) showing anammox microorganims in water samples from (A) coastal Africa, (B) the Black Sea, and (C) a bioreactor. (From Jetten, M.S.M., O. Sliekers, M. Kuypers, et al. 2003. Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. *Appl. Microbiol. Biotechnol.* **63**:107–114, fig. 2. With kind permission of Springer Science and Business Media.)

6 *2006. Community genomics (metagenome) approach develops blueprint for biochemical and genetic basis of anammox process*. DNA sequences from shotgun, fosmid, and bacterial artificial chromosome (BAC) clone libraries (see Section 6.9) are assembled from the mixed microbial community in an anammox bioreactor. Efforts to assemble the genome of the active bacterium, named *Cadidatus* Kuenenia stuttgartiensis, are incomplete; they stall at five large contigs (see Section 6.9), but information does include many insights into the overall metabolism and evolution of the organisms (Strous et al., 2006).

7 *2011. Community transcriptomics and proteomics (tied to isotopic tracers and physiological assays) elucidate detailed enzymatic machinery of anammox.* Hydrazine is produced from NO_2^- and NH_3 via the precursor NO (nitric oxide). Key enzymes catalyzing hydrazine synthesis and hydrazine oxidation to N_2 were purified. Novel N–N bond formation (between NO and NH_3) was demonstrated (Kartal et al., 2011) (Figure 2).

Box 7.8 *Continued*

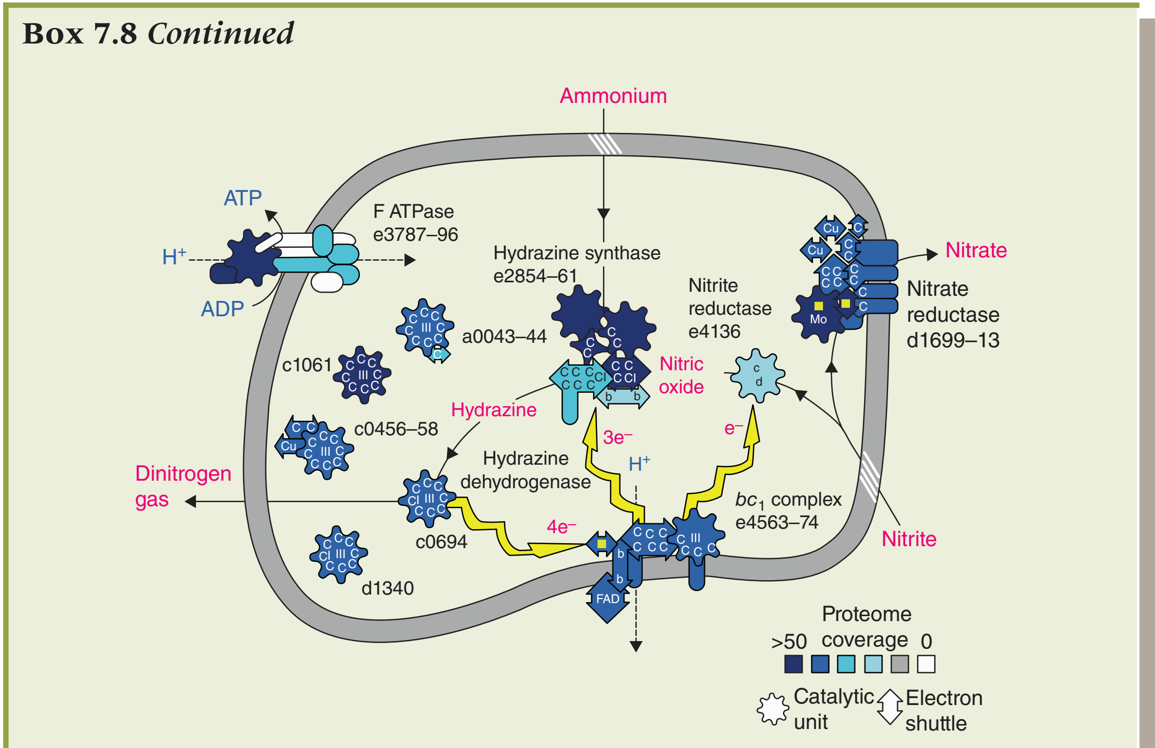


Figure 2 Biochemical pathway and enzymatic machinery for anammox reaction (carried out by *Candidatus* Kuenenia stuttgartiensis. The anammoxosome, an intracytoplasmic compartment bounded by a membrane (gray line), is the locus of anammox catabolism. Hydrazine synthase depicted in the center of the figure is loosely membrane associated. Yellow arrows, electron flow; yellow square, iron sulfur clusters; b, haem b; c, haem c; cl, atypical haem c; d, haem d; Mo, molybdopterin. (Reprinted by permission from Macmillan Publishers Ltd: Nature, from Kartal B, W.J. Maalceke, N.M. de Almeida, I. Cirpus, et al. 2011. Molecular mechanism of anaerobic ammonium oxidation. *Nature* **479**:127–130. Copyright 2011.)

that ammonium and methane were chemically inert under anaerobic conditions (Strous and Jetten, 2004).

As mentioned above, being able to cultivate (see Section 5.1) and use enrichment cultures (see Sections 6.2 and 6.11) are the gateways to discovering physiological and biochemical mechanisms in microorganisms. Pure cultures have not yet been isolated that are capable of anaerobically oxidizing either ammonium or methane; however, enrichment cultures are available for anammox and both sulfate- and nitrite- based AMO. Having active enrichment cultures available for laboratory-based inquiry makes a big difference in rates of discovery and scientific progress. The seven-step sequence of investigation for anammox benefited significantly from the availability of an engineered

Box 7.9

Timeline and events in the discovery of sulfate-based anaerobic methane oxidation (AMO): from field geochemistry to metagenomics

1. *~1957. Chemical oceanographers speculate*. Data sets from vertical sediment profiles show that methane and sulfate are simultaneously depleted in discrete zones in vertical sediment profiles.
2. *1979. Recognition of thermodynamic potential for CH_4 serving as e^- donor*. Early laboratory incubations led to the hypothesis "methanogenesis working in reverse in conjunction with a sulfate reducer". However, robust enrichment cultures are unsuccessful (Zehnder and Brock, 1979).
3. *1999. Use of field biogeochemistry and gene cloning*. Sediments above a methane hydrate deposit in the coastal Pacific Ocean have a lipid biomarker with a $^{13}C/^{12}C$ value that proves it was derived from the methane deposit on the ocean floor. The methane must have been the carbon source for the microorganisms. Clone libraries of 16S rRNA genes reveal several unusual sequences that suggest the identity of organisms involved in AMO (Hinrichs et al., 1999).
4. *2000. Fluorescent in situ hybridization (FISH) microscopy and radiotracer based physiology*. Microscopy (FISH) of field samples proves robust physical associations between cooperating methanogenic *Archaea* and sulfate-reducing *Bacteria*. Laboratory assays show that $^{35}SO_4$ is reduced to $H_2{}^{35}S$ (Boetius et al., 2000).
5. *2001. Confirmation of $^{13}C/^{12}C$ biomarker and FISH*. Spatially resolved mass spectrometry confirms that the field-derived associations of methanogens and sulfate reducers are composed of carbon whose $^{13}C/^{12}C$ ratio links them to in situ methane oxidation (Orphan et al., 2001).
6. *2002. Confirmation of biogeochemical significance: AMO creates large geochemical deposits in the Black Sea*. Large reefs (deposits of $Ca/MgCO_3$) at the bottom of the Black Sea were created from the CO_2 produced by AMO; evidence is supported by FISH, conversion of $^{14}CH_4$ to $^{14}CO_2$, and stable isotope ratios in both lipid biomarkers and the carbonate minerals (Michaelis et al., 2002).
7. *2003. Field enzymology*. Extracted protein from Black Sea reefs reveals an abundant nickel-containing enzyme that is closely related to enzymes in methanogenic *Archaea* (Krüger et al., 2003).
8. *2004. Community genomics (metagenomic) approach develops preliminary genetic blueprint for biochemical and genetic basis of process*. DNA sequences from shotgun and fosmid libraries (see Section 6.9) are assembled from a partially purified microbial community from the coastal Pacific Ocean. Partial assembly of genome fragments from two archaeal genomes and three sulfate reducers result. Identification of most of the genes associated with methanogenesis provides strong support for the "reverse methanogenesis" hypothesis (Hallam et al., 2004).
9. *2012. Key enzyme in methanogenesis pathway (methyl-coenzyme M reductase) is isolated from AMO Black Sea mats: crystal structure determined*. The structural differences between methyl-coenzyme M-reductase used in methanogenesis (by methanogens) versus reverse methanogenesis (by methanotrophic *Archaea*) were examined (Shima et al., 2012).
10. *2012. Cooperative metabolic pathway between sulfate-reducing bacterium and reverse-methanogenic Archaea elucidated.* After 8 years of cultivation, the highly enriched consortium was subjected to isotopic tracer, microscopic, and immunolabeling assays. The archaeal partner ("reverse methanogen") oxidizes methane and reduces sulfate to disulfide (S^0). The disulfide is exported, taken up, and disproportionated by the partner bacteria – producing sulfide and sulfate (Milucka et al., 2012) (Figure 1).

Box 7.9 ***Continued***

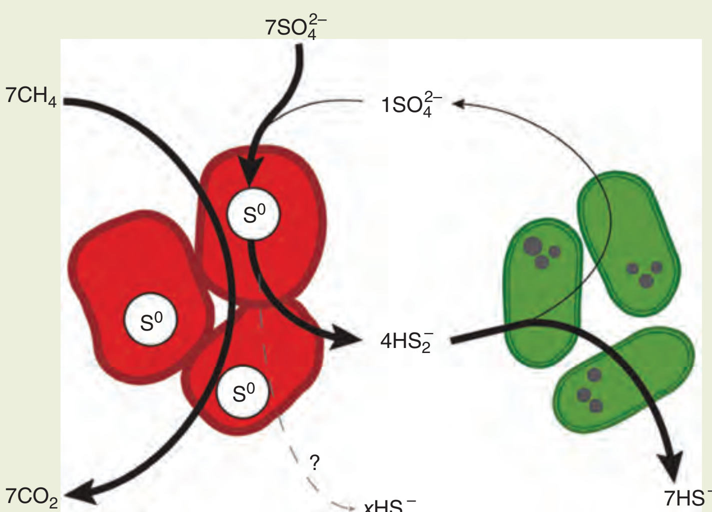


Figure 1 Model of anaerobic oxidation of methane coupled to sulfate reduction. Anaerobic methane-oxidizing *Archaea* (ANME) oxidize methane with a concomitant reduction of sulfate to zero-valent sulfur (S^0, elemental sulfur) that is partially deposited or bound intracellularly. Produced S^0 is exported or diffuses outside the cell where it reacts with sulfide to form polysulfides (disulfide, among others). Disulfide is taken up by the associated *Deltaproteobacteria* and is disproportionated to sulfate and sulfide. Sulfate produced during disproportionation might be re-used by the ANME and the ANME may also reduce some of the sulfate all the way to sulfide (grey dotted line). Dark circles in the bacteria represent intracellular precipitates rich in iron and phosphorus. (Reprinted by permission from Macmillan Publishers Ltd: Nature, from Milucka, J., T.G. Ferdelman, L. Polerecky, et al. 2012. Zero-valent sulphur is a key intermediate in marine methane oxidation. *Nature* **491**:541–546. Copyright 2012.)

bioreactor system in which the microbially mediated reaction between nitrite and ammonium could be carefully controlled and modified. The catalytic anammox activity could be transferred from one culture vessel to another. The growing biomass fixed CO_2 (was autotrophic) produced intermediary metabolites that could be traced and identified, and produced enzymes that mediated steps in the metabolic reaction pathway. Moreover, microscopic and biomarker (unique ladderane lipids and 16S rRNA sequence)-based characterizations were completed. Armed with biochemical fundamentals, investigators were able to explore the ecological significance of anammox. As mentioned in Section 7.4, anammox is currently thought to account for *at least* 30–50% of all marine ammonium oxidation.

The path of sulfate-based AMO discovery is shown in Box 7.9. Because laboratory-based enrichment cultures of sulfate-based AMO were extremely difficult to obtain, approximately half of the 10 steps shown in Box 7.9 were devoted to documenting AMO. All of the procedures used to document and biochemically characterize AMO began in anaerobic field-study sites that were highly enriched in methane (e.g., methane hydrate deposits in cold, deep Pacific Ocean sediments or in the Black Sea). A key factor in the logic of all the biomarker-based studies of AMO is the fortuitous fact that microbially produced methane has a unique and extremely negative $\delta^{13}C$ value (see Box 2.2). No other pools of carbon in nature are as depleted in ^{13}C as biogenic methane. Thus, a stable isotopic tracer experiment was built into the field-study sites! When isotope ratio mass spectrometery was used to analyze archaeal lipids extracted from sediments adjacent to the methane source, the $\delta^{13}C$ value of the lipids was extremely negative – this meant that the organisms in the sediments had to be using methane-derived carbon as their carbon source. Then, microscopy, fluorescent in situ hybridization (FISH), laboratory radiotracer studies (for both sulfate reduction and conversion of CH_4 to CO_2), and a sediment-derived enzyme all provided additional information about the sulfate-based AMO process (Box 7.9). Sulfate-based AMO is carried out by a pair of physiologically cooperating microorganisms: a "methanogen operating in reverse" which delivers disulfide to a bacterial partner (a Deltaproteobacteriium, closely related to sulfate reducers) that processes the disulfide it receives. It is estimated that 75% of all marine methane oxidation is attributable to sulfate-based AMO (Strous and Jetten, 2004; Milucka et al., 2012).

Astounding manifestations of resource exploitation by microbial life and evolutionary metabolic diversity continue to be uncovered. Anaerobic methane oxidation using *nitrite as an electron acceptor* (see Box 7.10) represents an additional scientific frontier. Microbial physiologists have speculated about the existence of pathways linking methane (as electron donor) to either nitrate or nitrite as electron acceptor (see Section 3.8) for decades. The first tangible evidence of this was reported by Raghoebarsing et al. (2006), who unequivocally demonstrated that an anaerobic enrichment culture from freshwater agricultural sediments produced N_2 gas while consuming methane. After applying three key omics methodologies (genomics, transcriptomics, and proteomics; see Section 6.10) to the active microbial consortium, Ettwig et al. (2010) made the shocking discovery that methane oxidation in this anaerobic culture follows the classic oxygen-dependent catabolic pathway (using methane monooxygenase). The active cells generated a small intracellular pool of O_2 (necessary for enzymatic attack of methane) via a novel pathway that consumes two molecules of nitric oxide (NO) and converts them to N_2 and O_2 via the newly discovered enzyme, nitric oxide dismutase (see Box 7.10).

Box 7.10

Timeline and events in the discovery of nitrite-based anaerobic methane oxidation (AMO): from enrichment cultures to novel biogeochemistry in the field.

1 *~2006. Laboratory enrichment of agricultural sediment proves nitrate/nitrite and methane are essential for growth of microorganisms*. As methane was consumed, nitrite was converted to N_2 gas. Sequencing of 16S rRNA genes indicated a dominant novel group of *Bacteria* and the abundance of the cells was confirmed microscopically with FISH (Raghoebaring et al., 2006).

2 *2010. Metagenomic assembly, metatranscriptomic and metaproteomic analyses and stable isotopic tracer assays of physiological reactions identify Candidatus* Methylomirabilis oxyfera *as the agent able to couple methane oxidation to nitrite reduction via a novel oxygen-producing pathway*. The draft genome for the dominant organism lacked genes for a complete denitrification pathway but, surprisingly, possessed a complete set of genes for *aerobic* methane oxidation. Authors discovered a novel oxygenic pathway that converts two NO molecules to N_2 and O_2 (via nitric oxide dismutase, which was previously unknown). The endogenously produced oxygen then reacts, via methane monooxygenase, with the methane substrate (Ettwig et al., 2010, 2012) (Figure 1).

3 *2013. Metaproteomic analysis of microbial community from a contaminated aquifer shows 8 of 9 proteins in the NO dismutation and methane-oxidation pathway to be expressed in situ* (Hanson and Madsen, 2015).

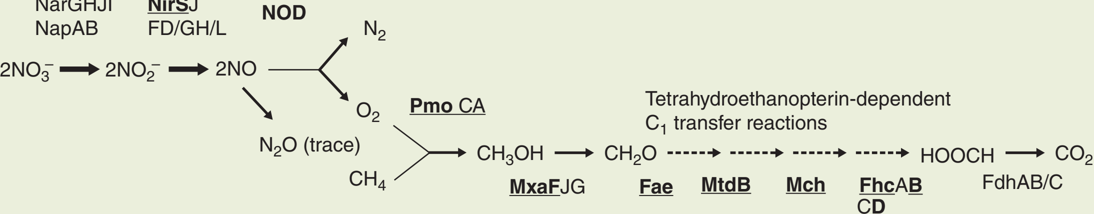


Figure 1 Pathway of nitrate-dependent anaerobic methane oxidation by *Candidatus* Methylomirabilis oxyfera. Genes are listed area as follows: *narGHJI*, nitrate reductase; *napAB*, periplasmic nitrate reductase; *nirSJFD/GH/L*, nitrite reductase; NOD (NO dismutase) is the key enzyme for carrying out the disproportionation of 2NO to $N_2 + O_2$;*nosDFYLZ*, nitrous oxide reductase; *pmoCA*, particulate methane monooxygenase; *mxaFJG*, methanol dehydrogenase; *fae*, formaldehyde-activating enzyme; *mtdB*, methylene-tetrahydromethanopterin (H_4MPT) dehydrogenase; *mch*, methenyl-H_4MPT cyclohydrolase; *fhcABCD*, formyltransferase/hydrolase; *fdhABC*, formate dehydrogenase. (Figure was assembled by B. Hanson, modified from Ettwig et al., 2010, 2012, and Luesken et al., 2012.)

7.6 MASS BALANCE APPROACHES TO ELEMENTAL CYCLES

In Section 7.4 we acknowledged that, for physiological and biochemical reasons, it can be insightful to conceptually split the elements away from one another. If we examine the "stuff of life" (the live and dead biotic components of the biosphere plus nutrients; Section 7.3) and sort through

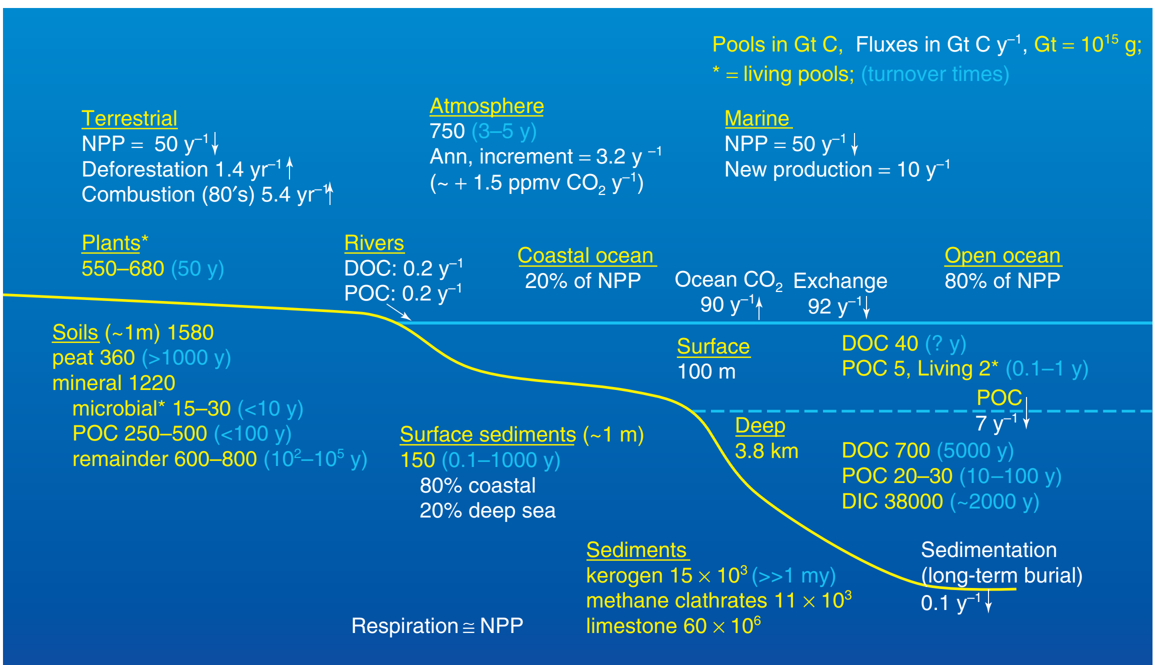


Figure 7.18 Global carbon reservoirs, fluxes, and turnover times. DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; NPP, net primary productivity; POC, particulate organic carbon; ppmv, parts per million on a volume basis. (From Reeburgh, W.S. 1997. Figures summarizing the global cycles of biogeochemically important elements. *Bull. Ecol. Soc. Am.* **78**:260–267. Reprinted with permission from the Ecological Society of America, http://www.ess.uci.edu/~reeburgh/fig1.html.)

these materials by elemental content, then budgets can be assembled. We have seen (Section 7.2) that such budgets help assess the status and health of Earth. These scientific exercises tally the sizes of nutrient pools and the fluxes of materials between them. Figures 7.18 to 7.20 portray global elemental cycles of carbon, sulfur, and nitrogen for terrestrial, atmospheric, and marine compartments of the globe.

Annual global carbon budget

The annual global carbon budget (Figure 7.18) provides insight into the function of the biosphere via the contrasting size and turnover times of the carbon pools. The current estimate of annual photosynthetic NPP (CO_2 fixation in excess of respiration) is between 50 and 60 × 10^{15} g C for both terrestrial and marine habitats (Reeburgh, 1997; Schlesinger, 1997). With the exception of prokaryotic biomass (see Section 4.5 and Table 4.10), the biomass of land plants (550–680 × 10^{15} g C) far exceeds that of any other biota and on a dry-weight basis plant biomass is ~50% cellulose and 15–36% lignin (see Section 7.3 and Box 7.3; Deobald and Crawford, 2002; Lynd et al., 2002). It is noteworthy that 20% of oceanic primary productivity is

concentrated along continental coastlines (see Section 4.3). The largest active pools of carbon in terrestrial and oceanic compartments are soil organic matter (viable organisms plus recognizable dead and decaying biomass plus humic substances) and dissolved organic carbon (DOC; Jiao et al., 2011), respectively. A small increase in the rate of microbial carbon mineralization from either of these two pools could significantly influence the concentration of atmospheric CO_2 (though this seems unlikely for deep oceanic DOC because of its slow turnover time of ~5000 years). Conversely, the stored soil and oceanic organic pools are so large that a small relative shift toward carbon storage (also termed sequestration) could possibly compensate for excesses in anthropogenic CO_2 emissions to the atmosphere.

Enormous additional pools of carbon, remote from the biosphere, occur in deep terrestrial and oceanic sites as keragen and methane hydrates (termed clathrates in Figure 7.18) and as carbonate minerals (limestone and dissolved inorganic carbon, DIC). Note that, in preparing Figure 7.18, Reeburgh (1997) explicitly showed an annual increase in atmospheric CO_2, as well as net absorption of CO_2 by ocean waters. Clearly the global carbon budget reveals a planet that is not in steady state. Moreover, the added CO_2 to ocean water has, via bicarbonate equilibrium reactions, the potential to cause gradual oceanic acidification. Additional information about the global carbon budget is available from Falkowski et al. (2000), Reay et al. (2008), and Houghton (2007).

Annual global sulfur budget

Highlighted in the global sulfur budget (Figure 7.19) are atmospheric transfers between terrestrial, oceanic, and atmospheric pools. As shown in Figure 7.19, terrestrial anthropogenic emission of sulfur to the atmosphere exceeds biogenic emissions by nearly a factor of four. The main sources of anthropogenic sulfur releases (largely as SO_2 gas) are coal burning and sulfide-ore smelting (Charlson et al., 2000). When SO_2 is emitted, it forms sulfuric acid by reactions with water in the atmosphere. Acid rain and its geochemical and biological effects are central concerns for understanding the global sulfur cycle.

Owing to its relatively long turnover time (5–10 years), carbonyl sulfide (COS, alternatively OCS) is the most abundant sulfur gas in the atmosphere (~500 parts per trillion). According to Schlesinger (1997) and Kettle et al. (2002) the major source of atmospheric COS is a photochemical reaction with dissolved organic matter in ocean water. Other COS sources include atmospheric oxidation of marine DMS (see Box 7.5), biomass burning, fossil fuel combustion, and atmospheric oxidation of industrially released carbon disulfide (CS_2). Schlesinger (1997) states that "our understanding of COS biogeochemistry is primitive". Like other sulfur-containing gases, COS is chemically oxidized to SO_4^{2-}, which participates in aerosol formation. The role of aerosols and cloud-induced changes in atmospheric reflectance of sunlight was previously discussed in Box 7.5. Atmospheric

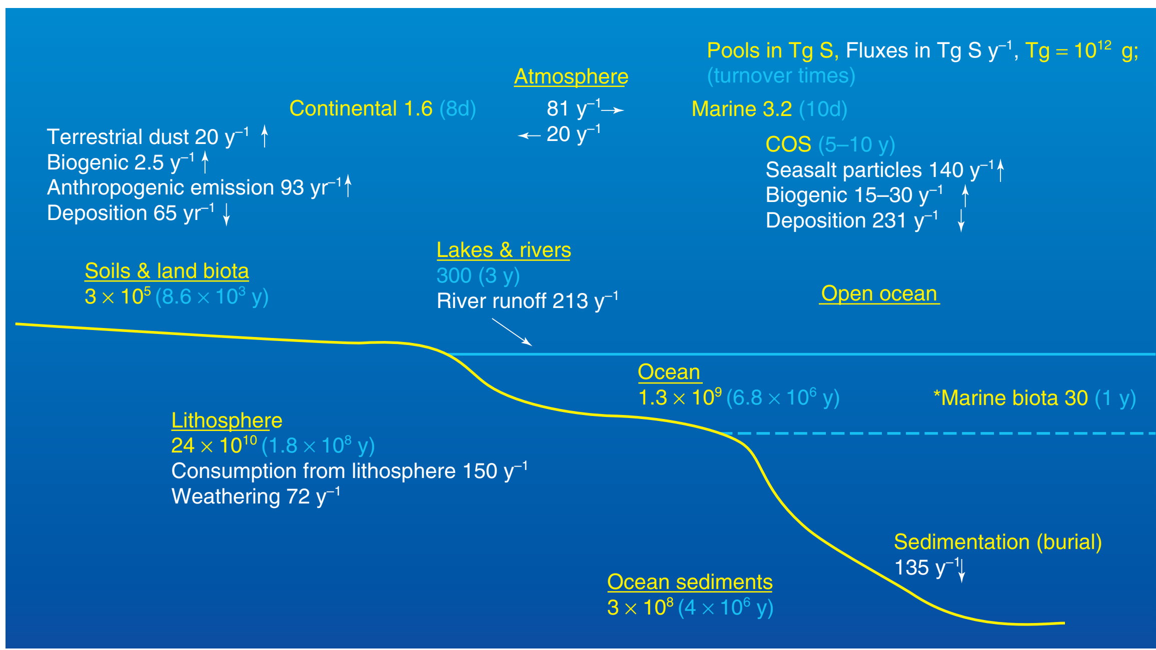


Figure 7.19 Global sulfur reservoirs, fluxes, and turnover times. (From Reeburgh, W.S. 1997. Figures summarizing the global cycles of biogeochemically important elements. *Bull. Ecol. Soc. Am.* **78**:260–267. Reprinted with permission from the Ecological Society of America, http://www.ess.uci.edu/~reeburgh/fig6.html.)

scientists (e.g., Wigley, 2006) have suggested large-scale engineered release of sulfate aerosol precursors into the atmosphere (simulating volcanic eruptions) as a means to offset global warming and to provide additional time to reduce human dependence on fossil fuels for energy. The major masses of sulfur in the global cycle are found in the lithosphere and oceanic compartments – largely as the crustal minerals gypsum ($CaSO_4$) and pyrite (FeS_2). Additional information about the global sulfur budget is available from Canfield and Farquhar (2012).

Annual global nitrogen budget

The annual global nitrogen budget (Figure 7.20) reveals an excess of ~100 × 10^{12} g nitrogen brought into the biosphere from the atmosphere, relative to the amount returned to the atmosphere via denitrification-related processes. The increases in nitrogen made available to terrestrial and aquatic biota are derived largely from industrial fertilizer production, enhanced biological nitrogen fixation in agriculture, and the burning of nitrogen-containing fossil fuels. These three sources boost background N_2 fixation via naturally occurring free-living and symbiotic prokaryotes by approximately two-thirds (Galloway et al., 2004, 2008). The environmental impacts of this imbalance in nitrogen cycling (summarized by Jaffe, 2000)

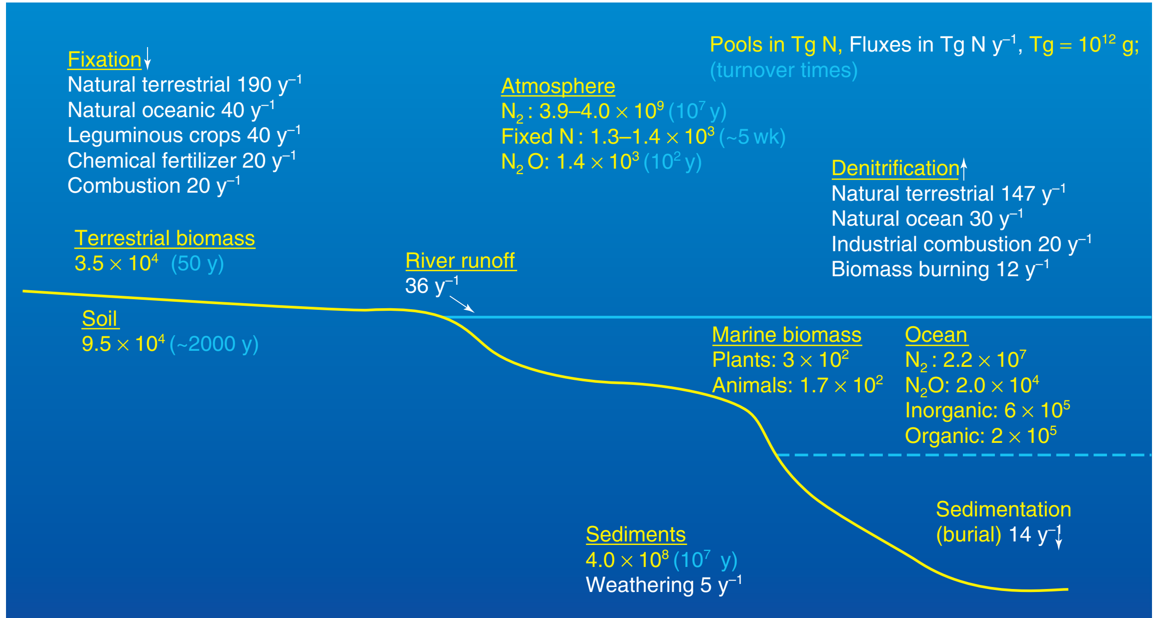


Figure 7.20 Global nitrogen reservoirs, fluxes, and turnover times. (From Reeburgh, W.S. 1997. Figures summarizing the global cycles of biogeochemically important elements. *Bull. Ecol. Soc. Am.* **78**:260–267. Reprinted with permission from the Ecological Society of America http://www.ess.uci.edu/~reeburgh/fig3.htm.)

include: (i) radiative forcing of climate change by N_2O and O_3 in the troposphere (see Section 7.2 and Box 7.7); (ii) photochemical smog; (iii) acid precipitation (deposition of nitric acid, HNO_3); (iv) stratospheric depletion of the ozone shield by N_2O reactions; (v) groundwater contamination by nitrate; (vi) fertilization of the global carbon cycle; and (vii) potential shifts in plant, animal, and microbial biodiversity in fertilized habitats. Additional information about the global nitrogen budget is available from Canfield et al. (2010a) and Reay et al. (2008).

The cycles of carbon, sulfur, and nitrogen have been disturbed by human activities. All are strongly influenced by transformations mediated by prokaryotes. Furthermore, advanced understanding of all three elemental cycles (and others) will be achieved via close collaboration between microbiologists, geochemists, atmospheric chemists, engineers, and modelers.

STUDY QUESTIONS

1 Data in Table 7.1 show the mass of mineral nutrients needed to produce 100 g dry mass of microbial cells. Table 7.2 and Box 7.6 both mention vanadium (V) as a potential nutrient required for nitrogen fixation. If you wanted to implement an assay showing that V is required as a nutrient, what three main considerations would be essential before implementing the experiment? (Hint: consider practical issues about the organism tested, its physiology, and background levels of V.)

2 Table 7.3, entry 3 lists a *cadA* gene (encoding the cadA protein) that detoxifies both Cd^{2+} and Zn^{2+} in bacterial cells. Venture a simple educated guess as to why the protein recognizes both Cd^{2+} and Zn^{2+}. (Hint: inspect the periodic table of the elements.)

3 CFC-11 and CFC-12 are mentioned in Section 7.2 and appear in Box 7.1 because of their influence on climate change as greenhouse gases in the troposphere. What other environmental process (not discussed here in this book) makes CFCs a major environmental concern? Where in the atmosphere does this process occur?

4 Figure 7.4 shows historical records of the rise in greenhouse gases in the troposphere. Carbon dioxide and methane fluctuate sinusoidally each year (Section 7.2). CFC-12 and CFC-11 do not fluctuate annually. Why?

5 What is your personal view of global warming? In preparing your answer, consider historical, geologic, and biological observations and evidence near your home and abroad. Which four of the many factors influencing global warming (Figure 7.5) do you think are most important? Why?

6 Construct three sketches of carbon budgets for the place you live. Carry out this exercise at the simplest level (your body) and two progressively higher levels, such as your home, your apartment complex, your neighborhood, your village, region, and/or state. Draw boxes and label the carbon pools. Draw arrows and label the processes. Note your major uncertainties in each model. What happens as the size of the system of interest increases?

7 Table 7.4 and Box 7.3 convey information about high molecular weight carbon compounds and how they are metabolized by microorganisms.
   (A) Name two major structural differences between starch molecules (that *you* digest) and cellulose molecules (that bacteria and fungi digest).
   (B) In both humans and fungi, what is the endproduct of hydrolytic digestion of starch and how is it further metabolized?
   (C) What sets lignin apart from the other biopolymers shown in Table 7.4 and Box 7.3? Briefly describe lignin formation and biodegradation.

8 Forest trees are fully biodegradable. Is there a forest or woodland or park near your home?
   (A) Visit the forest and take notes on the degree of decay in the dead wood.
   (B) Can you see fungal hyphae? Mycelia? Fruiting bodies?
   (C) What is the evidence for decay?
   (D) How long does it take for decay to be complete? Venture a guess. Also, use information in Chapter 6 to design one or more experiments to answer the question.

9 Information in Section 7.3 (including Table 7.4 and Figures 7.10 to 7.12) describes microbial metabolism of petroleum hydrocarbons.
   (A) Summarize the biochemical “logic” (the physiological strategy) for destabilizing the aromatic ring of a molecule under both aerobic and anaerobic conditions.
   (B) There are structural similarities between aromatic compounds in petroleum and in lignin. In light of your answer to question 8D, how long do you think it takes naturally occurring microorganisms to biodegrade the aromatic (and other) components in spilled crude oil? Please answer this by consulting library sources describing the Exxon Valdez, the 2010 Deepwater Horizon Gulf oil spill, or other oil spills.

10 The vertical scale in Table 7.5 associates being reduced with serving as an electron donor and being oxidized with serving as an electron acceptor. Tetrachloroethene ($Cl_2C{=}CCl_2$) is a widely used industrial solvent in the metal machining and electronics industries. Tetrachloroethene (also known as perchloroethene, or PCE) is also a widespread groundwater pollutant.
   (A) What is the oxidation state of carbon in PCE?
   (B) What is the physiological role you expect PCE to play in microbial metabolism?
   (C) If you were planning biodegradation tests for PCE, what experimental design would you use? What would you measure? Under what conditions would biodegradation occur?

11 Focus on the DMS and DMSO entries in Table 7.4.
- (A) Diagram the DMS–DMSO redox cycle by placing DMS on the right-hand side, DMSO on the left-hand side, and adding semicircular arrows to connect them (one arrow above and one arrow below). Label the top of the diagram "aerobic" and the bottom "anaerobic".
- (B) In a marine setting, what would be the likely electron donors and acceptors to drive the cycle?
- (C) Consider where DMSO falls on the redox scale in Table 7.5 and in Figure 3.10. What physiological or ecological circumstances would favor DMSO utilization as an electron *donor* rather than an electron *acceptor*?

12 Scrutinize Figure 7.14.
- (A) Were you aware of the detritus-based food chains prior to reading Section 7.4?
- (B) Choose a habitat of interest within your favorite ecosystem. Next, state the resources and organisms likely to be participating in the detritus food chain in that habitat.
- (C) As a related library research task, search for information on the "microbial loop". What did you find? Research papers using this term attest to its scientific significance, especially in aquatic systems.

13 In depicting the carbon cycle, Figure 7.15 includes a pathway "burial → (diagenesis) → peats, lignites". Likewise, Figure 7.18, in depicting the global carbon budget, includes "sedimentation".
- (A) Does this mean that fossil fuels are renewable?
- (B) Could global warming be corrected by "sequestering" or removing atmospheric CO_2 from the biosphere? Develop microbiological, geochemical, and/or engineering arguments to support your answer.

14 Consider the nitrogen cycle as described in Sections 7.3, 7.4, and 7.6 (as supported by information in Tables 7.4 to 7.6, Figures 7.17 and 7.20, and Boxes 7.6, 7.8, and 7.10).
- (A) If you were writing a science fiction novel, would you have invented a biogeochemical cycle more complex than the nitrogen cycle? Does it seem "stranger than fiction" to you?
- (B) What does the complexity of pathways and microbial metabolic adaptations say about the role of nitrogen compounds in evolution (consider, especially, N_2 fixation)?
- (C) The text of Box 7.6 provides a fortuitous physiological fact that has led to a widespread assay for determining nitrogenase activity in both microbial cultures and in environmental samples. What is that assay? Why is it useful and important?
- (D) Summarize the multiple connections between nitrogen biogeochemistry and climate change.
- (E) Prepare a diagram of the nitrogen cycle that is analogous to the one shown for the carbon cycle (Figure 7.15) and to the diagram you prepared in response to question 11A. Place ammonia on the far right and nitrate on the far left of the central horizontal line that divides processes into aerobic (top) and anaerobic (bottom). Now use this framework to create a unified conceptual summary of the nitrogen cycle – specifying key nitrogen compounds and the processes (e.g., nitrification, denitrification, nitrogen fixation, etc.) that connect them.

15 In reflecting on material presented in Section 7.5, consider parallels between discovering an AIDS vaccine and understanding mechanistic details of reactions that microorganisms carry out on the seafloor. Anammox (Box 7.8) and sulfate-based AMO (Box 7.9) are key biogeochemical processes that influence the "health" of the biosphere.
- (A) If environmental and physiological conditions diminished sulfate-based AMO activity on the seafloor, what climate change scenario might develop?
- (B) Likewise, if anammox activity on the seafloor diminished, what climate change scenario might develop?

In developing answers, consider the intricacies of direct effects, indirect effects, elemental mass balances (e.g., Figures 7.18 to 7.20), interactions between elemental cycles, and potential compensatory processes.

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8

Special and Applied Topics in Environmental Microbiology

As stated in Chapter 1, the core discipline of environmental microbiology centers on a "house-like" structure in which "evolution" is the base, "thermodynamics" and "habitat diversity" are the walls, and in which "physiology" and "ecology" serve as the two roof pieces. The house has gradually been built in Chapters 1 to 7. Here, we reach from the discipline's core foundations to special and applied topics.

First and foremost in extending environmental microbiology's core is the need to recognize other organisms *(both microorganisms and higher eukaryotes) as significant biogeochemical and evolutionary forces for life on Earth. Striking evolutionary adaptations reflecting relationships* between organisms *have developed. In this chapter, we cover fundamental principles of ecological relationships and then focus on the relationships of microorganisms to plants and humans. Furthermore, because the major environmental microbiological questions (who? what? when? where? how? and why?) have been asked (see Chapters 1 to 7), we can now use the answers and the methods of inquiry to address important topics in engineering (biodegradation and bioremediation), natural history (biofilms, evolution of metabolic pathways), biotechnology (energy production, genetically engineered crops), and medicine (emerging human pathogens, antibiotic resistance). Case studies are featured prominently in the sections focusing on the evolution of metabolic pathways and environmental biotechnology.*

Chapter 8 Outline

8.1 Other organisms as microbial habitats: ecological relationships
8.2 Microbial residents of plants and humans
8.3 Biodegradation and bioremediation
8.4 Biofilms
8.5 Evolution of catabolic pathways for organic contaminants
8.6 Environmental biotechnology: overview and nine case studies
8.7 Antibiotic resistance

8.1 OTHER ORGANISMS AS MICROBIAL HABITATS: ECOLOGICAL RELATIONSHIPS

Resource exploitation has been a major theme of this book. By understanding the materials and conditions that constitute selective pressures in a given habitat, we can predict and discover novel microbial processes and the organisms that carry them out. During early planetary history, prokaryotes were the sole forms of life (see Chapter 2) and the key selective pressures were geochemical ones found in ancient oceans (Figure 8.1, bottom).

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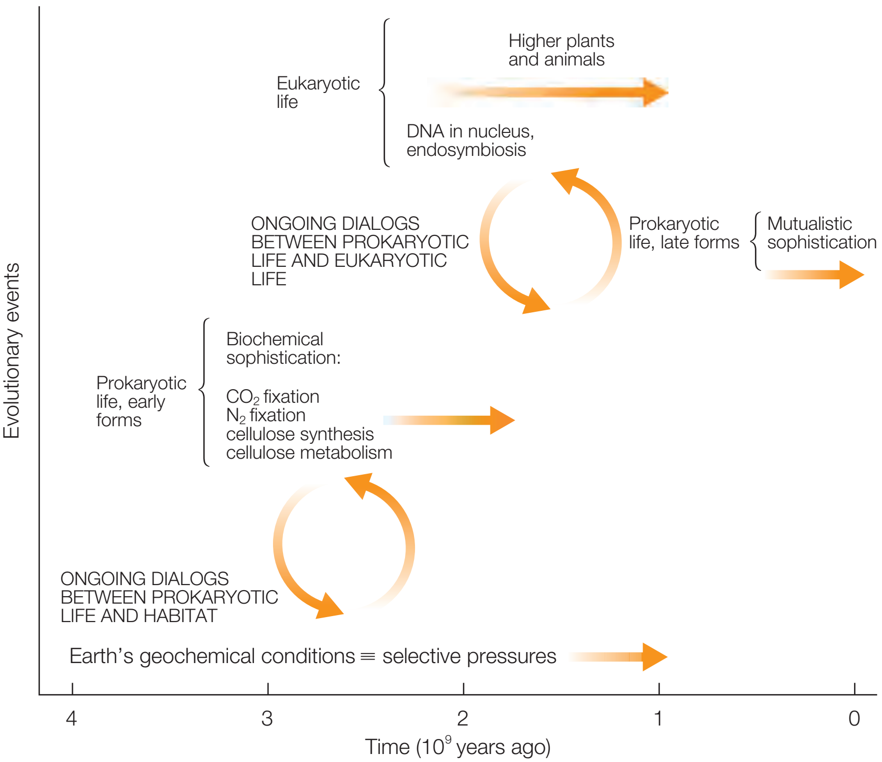


Figure 8.1 Sequence of evolutionary events leading to key mutualistic interactions between prokarytoic and eukaryotic forms of life.

As large, complex eukaryotic creatures (especially plants and animals; McFall-Ngai et al., 2013; Rosenberg and Zilber-Rosenberg, 2013) developed, these life forms, themselves, became new habitats that offered exploitable resources (Figure 8.1, top). It is clear, then, that *inner and outer compartments of other organisms* have played important roles in the evolution of members of the microbial world. In Chapter 7 (especially Section 7.3, Table 7.4), we focused upon resources available in *deceased biomass*. Here, in this section, we develop an overview of the many types of mutual adaptations that can arise between microorganisms and their *viable partners*. In their recent overview of interactions between higher animals and *Bacteria*, McFall-Ngai et al. (2013) emphasize that physiological, developmental, ecological, and genomic interactions between "higher" forms of life and microorganisms are extremely intricate and vastly unexplored.

The spectrum of ecological relationships between organisms

Because prokaryotes preceded eukaryotes as Earth's inhabitants, eukaryotes have always had prokaryotic partners (Figure 8.1). The relationships between organisms span a broad range and the impacts on one partner can be very different from those on the other partner. Figure 8.2 provides an

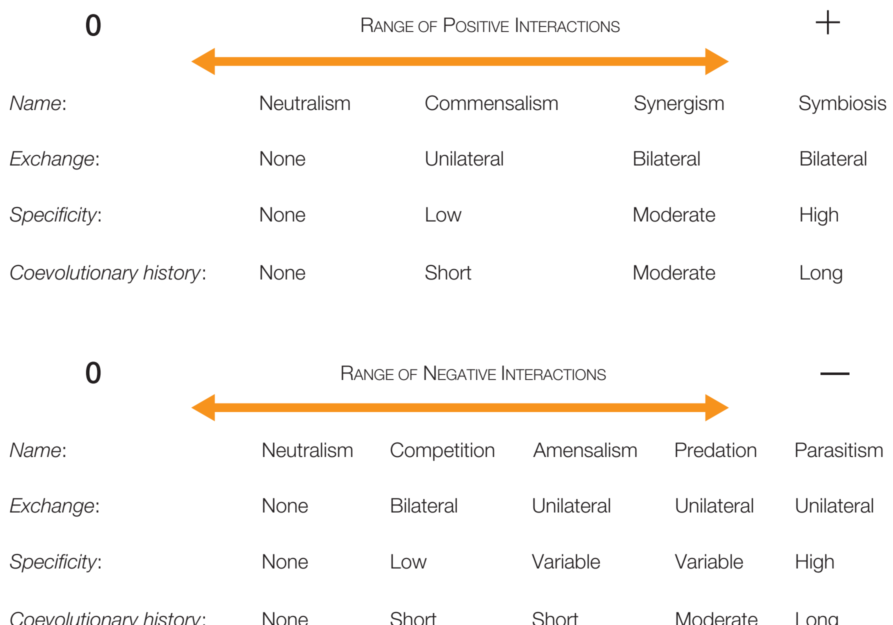


Figure 8.2 Spectrum of ecological interactions between two organisms. Shown are eight key categories of interaction and the degree of evolutionary dialog for each.

overview of the types of effects that organisms can have upon one another (from neutral to positive and neutral to negative). Ecologists have developed specific terminologies for describing the degree of positive or negative interaction and which partner is impacted.

At the mid-point between the extremes of positive and negative interactions is "neutralism" (listed twice in Figure 8.2, on the left side; McFall-Ngai et al., 2013). Neutralism represents the state in which organisms fail to interact with one another. From a microbial ecology perspective, neutralism is a rare situation that occurs in new, barely colonized habitats (such as recently emerged volcanic islands) or in habitats whose nutrient contents are so low that slow metabolism or dormancy predominates as a lifestyle. Box 8.1 provides information that elaborates upon the eight categories of ecological relationships that extend from symbiosis (also known as mutualism, at the positive extreme) to parasitism (at the negative extreme). Competition, amensalism, and predation are the intermediary categories of negative interaction. Commensalism and synergism (also known as proto-cooperation) are the intermediary categories of positive interaction. Key aspects of all eight categories include the type of exchange (one way or two way), the specificity of the relationship, and the extent of shared coevolutionary history of the organisms (Figure 8.2). In general, stable, nutrient-rich habitats are the sites where diverse, highly specialized biota are likely to evolve. These stable habitats are also sites where coevolution of symbiotic and parasitic ecological relationships are likely to occur.

Box 8.1

Eight categories of ecological relationships ranging from positive (symbiosis) to negative (parasitism) with characteristics and examples

Type of interaction	Category	Characteristics	Examples/habitats
None	Neutralism	Lack of interactions; low population densities; low growth rates; aliens in foreign habitats	Deserts, atmosphere, snow, ice, glaciers
Positive	Commensalism (mesa = table = table scraps; low specificity)	One-way positive exchange; one organism benefits from another's "table scraps"	Algae and plants leak carbon (photosynthate) to heterotrophs; methanogens provide methane to methanotrophs
	Synergism (also "proto-cooperation"; moderate specificity)	Two-way positive exchange; mutually beneficial to both partners	An alga provides carbon to epiphytic heterotrophs that synthesize a B vitamin required by the alga; hydrogen consumption by methanogens drives fermentation reactions in anaerobic food chains
	Symbiosis (also "mutualism"; high specificity)	Two-way positive exchange that allows a new biological trait to be expressed; this may be an obligate relationship; symbionts live singly in a different way than when together	*Rhizobium*–legume symbiosis, lichens, corals, ruminant animals, hydrothermal vent animals
Negative	Competition (low specificity)	Organisms compete for nutrient-limiting resources	In soil, sediment, and waters; many nutrients (e.g., C, N, P, Fe), electron donors, and electron acceptors may be limiting
	Amensalism (variable specificity)	One-way negative exchanges; one organism is antagonistic to another, often by releasing inhibitory chemicals	Antibiotic compounds from one microbe may prevent growth of a neighbor; in wine, ethanol produced by yeast inhibits bacterial growth; organic acids released by some anaerobes inhibit growth of adjacent populations
	Predation (variable specificity)	Big eats small; prey is engulfed; feeding is abrupt	Density-dependent feeding by protozoa on bacteria in soil and sediments; nematode-trapping fungi in soil; *Bdellovibrio* bacterium that attacks, penetrates, and consumes other bacteria
	Parasitism (high specificity)	Little debilitates big over prolonged period; parasite may require host (obligate) or be able to survive in absence of host (facultative); relationship may be balanced or destructive	Virus infections of microorganisms and animals

A survey of symbioses and their characteristics

There are at least three main questions to answer in attempting to understand symbioses:

1. What are their key features in terms of participants and resources exchanged?
2. How did symbioses develop over evolutionary time?
3. What are the detailed genetic and biochemical signaling pathways that allow contemporary symbioses to form and be maintained?

Only the first of these three questions will be addressed extensively in this section. Regarding the evolutionary development of symbioses, it is reasonable to speculate that an initially commensalistic relationship might later become synergistic and then symbiotic (see Figure 8.2 and Box 8.1). This progression from casual exchange to highly specialized, even obligate, metabolic function is likely to have evolved over time via a series of genetic and biochemical adaptations of one organism to the other. Contemporary symbiotic systems are endpoints of evolution that exhibit sophisticated biochemical signaling pathways that lead to the establishment of successfully functioning symbioses (Hoffmeister and Martin, 2003; McFall-Ngai et al., 2013). Analogous to our own immune system, a host of a symbiotic relationship must recognize the partner – distinguishing the partner from other microorganisms that may be invaders, pathogens, or parasites. A glimpse into the biochemical and genetic intricacies of the early steps of one symbiosis (*Rhizobium*–legume, described below) is presented in Box 8.2. Signaling molecules, known as "nod factors", are produced by the symbiotic bacteria. These interact specifically with membrane protein receptors at the surface of legume roots and the interaction causes structural alterations of root hairs, eventually leading to the formation of nitrogen-fixing root nodules.

At least three key physiological traits make some prokaryotes desirable symbiotic partners (Figure 8.1):

1. Autotrophy – especially CO_2 fixation linked to chemolithotrophic metabolism, such as sulfide oxidation (for background, see Sections 3.3 and 7.4).
2. Nitrogen fixation – a uniquely prokaryotic trait that is predominantly expressed at very low oxygen concentration and converts atmospheric N_2 to ammonia and amino acids (for background, see Sections 7.4 and 7.5).
3. Digestion of complex high molecular weight organic compounds – particularly cellulose and other plant polysaccharides (for background, see Section 7.3).

One or more of these traits contributes to the (often obligate) symbiotic relationships formed among microorganisms and between microorganisms and animals. By merging their genetic and biochemical traits, the two symbiotic partners achieve physiological abilities and remarkable lifestyles that neither can achieve alone.

Box 8.2

Early biochemical and genetic details of recognition and root-hair curling essential for the successful establishment of *Rhizobium*–legume symbiosis

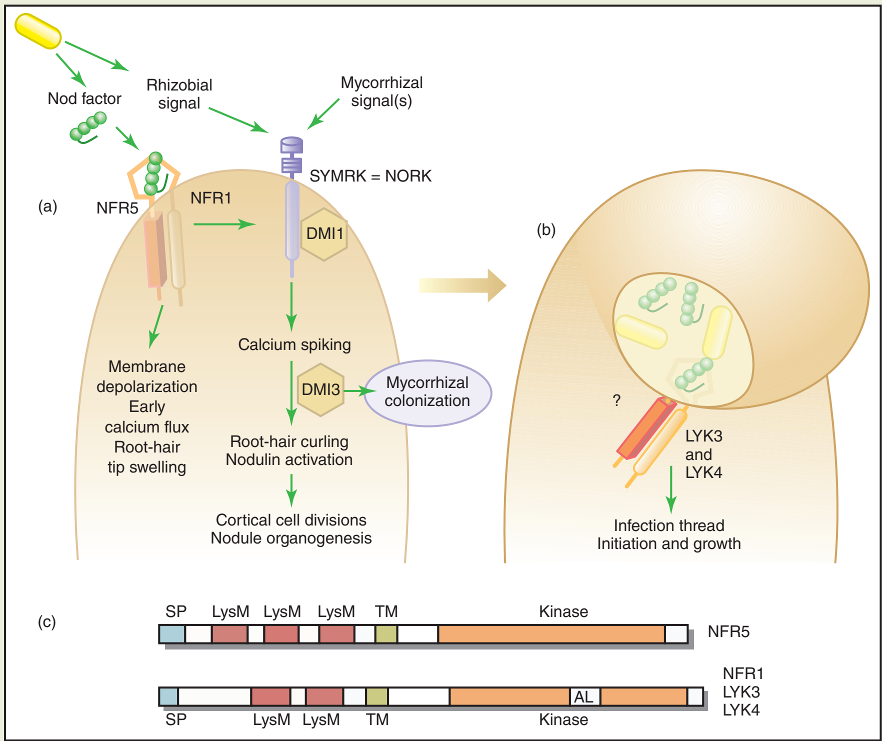


Figure 1 (a) Symbiotic rhizobial bacteria secrete Nod factors that are perceived by two linked transmembrane protein receptors (referred to as NFR1 and NFR5). The activated NFR1–NFR5 receptor initiates rapid calcium influx and swelling of root-hair tips, which are early events in the plant symbiotic response that may be specific for bacterial symbionts. Activation of the receptor is also required to activate another protein receptor complex (NORK–DMI1), which results in plant symbiotic responses to both bacterial and fungal symbionts. The NORK–DMI1 complex may also be involved in direct recognition of rhizobial and mycorrhizal signals. (b) Rhizobial bacteria entrapped in a curling root hair. In order for rhizobia to enter root hairs and to initiate the formation of infection threads and nodulation, the Nod factors they release must be recognized by highly specific plant receptors. Another set of membrane protein receptors (LYK3 and LYK4) of the plant host are involved in Nod factor recognition. (c) Schematic map of the many functional domains within the protein receptors (NFR5, NFR1, LYK3, and LYK4) in the nitrogen-fixing plant hosts. NFR5 has three LysM domains and its kinase domain does not contain an activation loop. NFR1, LYK3, and LYK4 have two LysM domains, and their kinase domains contain an activation loop. AL, activation loop in the kinase domain; SP, signal peptide; TM, transmembrane domain. (From Cullimore, J. and J. Denarie. 2003. Plant sciences: how legumes select their sweet talking symbionts. *Science* **302**:575–578. Reprinted with permission from AAAS.)

Table 8.1 provides an overview of many of the known types of symbiotic relationships, and their essential characteristics. Entries 1 and 2 of Table 8.1 remind the reader that the ability to hydrolyze cellulose is lacking in all animals higher on the evolutionary scale than mollusks (with the exception of silverfish (*Lepisma lineata*); Schlegel and Jannasch, 2006). Cellulose synthesis evolved in prokaryotes (Nobles and Brown, 2004); therefore they are the key agents of cellulose biodegradation (see Figure 8.1). Our world would be a very different place without the cellulose metabolism carried out by anaerobic microbial communities in specialized organs in the gastrointestinal tract of animals. These communities are essential for the animals' normal nutrition, which absorbs organic acid fermentation products through intestinal walls. If these microbial communities vanished, the global biogeochemical cycling of carbon would be drastically inhibited and many animals critical for human nutrition and commerce (cows, sheep, goats, horses, camels) would starve.

The next four entries in Table 8.1 focus on the soil habitat. For ~50 million years, tropical ants of the genus *Atta* have delivered leaf fragments to fungal gardens (entry 3; Figure 8.3). In these gardens, the fungi digest the leaf matter and the growing fungal biomass serves as food for the ants. An additional dimension of the symbiosis is the cultivation, in cavities within the ants, of bacteria that produce antibiotics used by the ants to keep parasites of the fungal garden at bay (Currie et al., 2006; Mehdiabadi et al., 2012).

Figure 8.3 Photograph of an ant of the genus *Atta* in its fungal garden. (Courtesy of Alex Wild Photography, www.myrmecos.net, with permission.)

Table 8.1

Examples of symbioses and their key characteristics

Entry	Habitat	Type of symbiosis	Partner A	Partner B	Beneficial resources exchanged between partners	References
1	Gastrointestinal tracts of animals	Ruminants (cows, sheep, goats, camels, giraffes) whose stomachs are extended at the entry point (foregut)	Herbivorous animal host with large stomach compartment known as the rumen. The animal is unable to digest cellulose and many other plant polysaccharides	Microbial community of anaerobic bacteria and protozoa	The animal provides a warm, well-mixed habitat periodically replenished with plant material. The microorganisms ferment the plant material into fatty acids absorbed through the animal stomach wall	Schlegel and Jannasch, 2006; Russell and Rychlik, 2001; Mizrahi, 2013
2	Gastrointestinal tracts of animals	Animals whose stomachs are modified near the exit point (hindgut). These include vertebrates (horses, pigs, rats, rabbits) and invertebrates (termites, wood roaches)	Herbivorous animal host whose intestinal tract features extended hindgut compartments (blind sacs, "ceca")	Microbial community of anaerobic bacteria and protozoa	The animal provides a warm, well-mixed habitat periodically replenished with plant material. The microorganisms ferment the plant material into fatty acids absorbed through the animal stomach wall	Schlegel and Jannasch, 2006; Isaacson and Kim, 2012
3	Soil	Ant–fungus	Attine leaf-cutting ants of the tropics (e.g., genus *Atta*)	Fungi in the family *Lepiotaceae*	The ants carefully create, maintain, and disseminate fungal gardens that serve as the ant's main food source. The fungi are fed and digest leaf material delivered by the ants; they also protect the ants from competitors and parasites	Currie, 2001; Currie et al., 2003, 2006; Mehdiabadi et al., 2012

Table 8.1 *Continued*

Entry	Habitat	Type of symbiosis	Partner A	Partner B	Beneficial resources exchanged between partners	References
4	Soil	*Rhizobium*–legume	Leguminous plants, ranging from arctic annuals to tropical trees	Bacteria of the genus *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*	Specialized organs (nodules) form on roots and stems of plants. These are composed of plant and bacterial tissue. Photosynthetic carbon from the plant is exchanged for fixed nitrogen from the bacterium	Van Rhijn and Vanderleyden, 1995; Perret et al., 2000; Geurts et al., 2012; Sadowsky et al., 2013
5	Soil	Mycorrhizae (arbuscular; endo)	Most vascular plants, including representatives of angiosperms, gymnosperms, and bryophytes	Many soil fungi (especially *Glomus*)	Fungi inhabit root cortical cells and obtain carbon from the plant. Fungi deliver mineral nutrients from soil to the root cortical cells	Harrison, 2005; Seguel et al., 2013
6	Soil	Mycorrhizae (ecto)	Many trees and shrubs of temperate regions	Many soil fungi, including *Basidiomycetes* and *Ascomycetes*	Resource exchange is like that of arbuscular mycorrhizae; however, in ectomycorrhizae, the root is enveloped in a fungal network; root tissue cells are surrounded, not penetrated	Martin et al., 2001; Nehls et al., 2010
7	Plant, rock, and soil surfaces	Lichens	Fungi	Algae (often cyanobacteria)	Close interweaving of fungal and algal tissues. The fungi provide a physical anchor, mineral nutrients, and desiccation resistance. The algae provide carbon (through photosynthesis) and nitrogen (if a cyanobacterium)	Nash, 1996; Adams et al., 2013; Kaasalainen et al., 2013

8	Tropical wetlands	*Azolla–Anabaena*	*Azolla* (a fern)	N_2-fixing cyanobacterium, *Anabaena*	The small floating fern provides cavities within its leaves that serve as a habitat for the cyanobacteria. The cyanobacterium fixes nitrogen in this intracellular association	Adams et al., 2013; Carrapiço, 2010
9	Rice plants	Fungus–bacterium in rice-blight disease	*Rhizopus*, plant pathogenic fungus that attacks rice	Intracellular bacterium	Fungus, long thought to be responsible for the plant disease, is pathogenic because endosymbiotic bacteria synthesize rhizoxin	Partida-Martinez and Hertwick, 2005; Scherlach et al., 2013
10	Ocean hydrothermal vents	*Riftia* worms	*Riftia* worms that lack a gastrointestinal tract	Sulfide-oxidizing chemolithoautotrophic bacteria	Specialized organ in worm is colonized by the bacteria. Bacteria receive shelter and H_2S; the worm receives fixed carbon	Jeanthon, 2000; Cavanaugh et al., 2013; Beinart et al., 2013
11	Ocean hydrothermal vents	Large clam (*Calyptogena*)	Bivalve clam with gills colonized by H_2S-oxidizing bacteria	Sulfide-oxidizing chemolithoautotrophs	Endosymbiont bacteria provide carbon and energy. Host provides shelter and transport of resources	Jeanthon, 2000; Cavanaugh et al., 2013; Roeselers and Newton, 2012
12	Coral reefs	Coral reef-forming animals	Sea anemone-like anthozoans that secrete protective exoskeletons of calcium carbonate	Unicellular dinoflagellate marine algae, *Symbiodinium*	Anthozoans provide shelter for algal symbionts whose photosynthetic activity supports growth and maintenance of the host	Baker, 2003; Davy el al., 2012

Table 8.1 *Continued*

Entry	Habitat	Type of symbiosis	Partner A	Partner B	Beneficial resources exchanged between partners	References
13	Ocean waters	Luminous squid plus *Vibrio fischeri* bacteria	Squid, *Euprymna scolopes*, with bioluminescent light organ on head	*Vibrio fisheri*, a bioluminescent bacteria	Specialized cavity provides housing and nutrients for bacteria whose ability to produce light assists the squid in avoiding predators	Ruby, 1996, 1999; Nyholm and McFall-Ngai, 2004; Lupp and Ruby, 2005; Altura et al., 2013
14	Ocean floor, whale fall	*Osedax* bone-eating worm	Mouthless, gutless polychaete worm that decomposes whale bones	*Oceanospirillales*, bacteria	Adult worms possess elaborate posterior root-like extensions that penetrate bones. Bacteria are thought to digest collagen- and cholesterol-rich tissues in exchange for shelter	Goffredi et al., 2005; Verna et al., 2010
15	Marine invertebrates	Shipworms	Wood-boring marine bivalve worms of the family *Teredinidae*	Cellulytic, nitrogen-fixing bacteria, *Terredinibacter turnerae*	Worms burrow into and consume floating or submerged wood; bacteria that colonize worm gills receive cellulose and shelter in exchange for cellulose digestion and amino acids	Felbeck and Distel, 1992; Distel et al., 2002; Elshahawi et al., 2013

16	Insects	Intracellular obligate endosymbioses of internal structures, termed “bacteriocytes”, associated with gut, ovary, and other insect organs	Several large orders of insects including aphids, psyllids, whiteflies, mealy-bugs, tsetse flies, weevils, carpenter ants, and termites	Many are members of γ- and β-Proteobacteria	The bacteria receive food and shelter; they dwell within clusters of structures, termed “bacteriocytes” inside the insect. Specific functions of insect–endosymbiont partnerships remain unknown in most cases. Main benefit to hosts is probably correction of dietary imbalances by synthesis of growth factors such as amino acids. Other benefits may include resistance to parasitism and broader food source utilization	Wernegreen, 2002; Scarborough et al., 2005; Baumann et al., 2006, 2013; Koga et al., 2012
17	Protozoa	Protozoa–intracellular symbiont	Many types of amoebae, flagellates, and ciliates	Many types of bacteria	By colonizing the interior of protozoa, bacteria receive food and shelter. If the bacteria give nothing back in return, they are parasites. However, positive effects range from synthesis of growth factors to providing electron sinks for anaerobic energy metabolism, to conferring selective advantages such as defense against predators	Horn and Wagner, 2004; Görtz, 2006; Schweikert et al., 2013

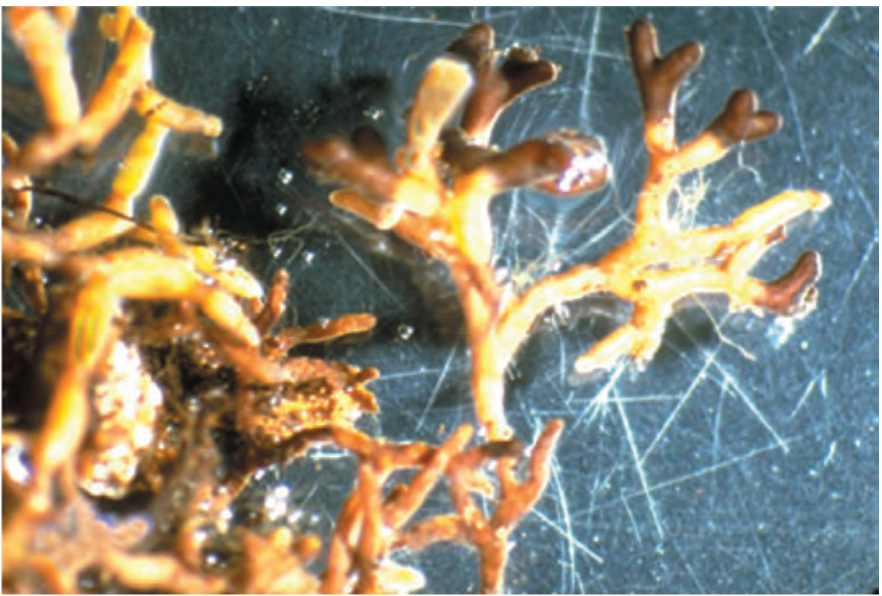


Figure 8.4 Photograph of the root of a pine seedling showing an ectomycorrhizal fungal sheath formed by *Amanita muscaria*, a Basidiomycete. Magnification approximately ×24. (Courtesy of R. Molina, US Forest Service, with permission.)

The *Rhizobium*–legume symbiosis (entry 4 of Table 8.1, see also Box 8.2) is a remarkably intricate cooperative effort between bacteria and plants: within specialized nodules, oxygen tension is buffered at very low levels, allowing expression of bacterial nitrogen-fixation genes and delivery of fixed nitrogen to the plant in exchange for refuge and carbon.

Mycorrhiza is a term literally meaning "fungus root". This commingling of tissues from two organisms, the mycorrhizal symbiosis, is an essential feature of the biology and ecology of the majority (>80%) of terrestrial plants. In this mutually beneficial relationship, the propensity of fungal hyphae to explore and exploit soil nutrients augments the plant root's own role in nutrient and water uptake. Furthermore, the fungal endosymbiont renders the plant less susceptible to infection by pathogenic fungi. In return for these services, the fungus receives plant photosynthate as a carbon source. When examined under a microscope, mycorrhizae exhibit two major morphological types. In the arbuscular form (also known as "endo", entry 5 of Table 8.1), the fungal filaments occur as inter- and intracellular coils and tree-like "arbuscules", largely within the body of the root. By contrast, the ectomycorrhizal morphology found on roots of many coniferous trees (entry 6; Figure 8.4) features an extensive hyphal sheath that coats the exterior of the root surface. Other subclasses of the symbiosis are

Figure 8.5 Photograph of the small water fern, *Azolla*, which houses within its tissue the nitrogen-fixing endosymbiont *Anabaena*. (Courtesy of Kurt Snyder and Wikipedia, with permission.)

recognized based largely upon the taxonomy of the host plant – especially orchyd, ericoid, arbutoid, and monotropoid mycorrhiza (Martin et al., 2001; Nehls et al., 2010).

Fungi also play a vital role in lichen symbiosis (Table 8.1, entry 7), which relies upon intimate physical and nutritional linkages between the fungal heterotroph and a photosynthetic autotroph (either eukaryotic algae or prokaryotic cyanobacteria). Together, the two lichen partners are able to colonize harsh, dry, low-nutrient habitats (rocks, tree surfaces, facades of buildings) – cooperatively growing and reproducing by scavenging water, light, and minerals. The symbiosis between the tropical fern (*Azolla*) and cyanobacterium (*Anabaena*) (Table 8.1, entry 8; Figure 8.5) has been exploited by rice farmers for centuries as a way to boost the nitrogen status of rice paddies. The final plant-related entry in Table 8.1 (entry 9) is the recently discovered relationship between an intracellular bacterium that colonizes the plant-pathogenic fungus, *Rhizopus*, responsible for rice blight disease. Remarkably, the key toxin that confers virulence on the fungal pathogen is synthesized by the endosymbiotic bacterium. This new information invites new strategies for controlling the plant disease: inhibit the bacterium that inhabits the fungus.

Figure 8.6 Photograph of the squid, *Euprymna scolopes*, which features a light organ colonized by the bacterium *Vibrio fischeri*. (Courtesy of C. Frazee and M. McFall-Ngai via E.G. Ruby, University of Wisconsin, with permission.)

The next six entries in Table 8.1 make it clear that marine habitats support a wide diversity of symbiotic relationships. On the dark ocean floor where hydrothermal vents release hot sulfide-rich water, there are oases of life supported by sulfide-oxidizing chemolithotroph bacteria. Two important animal species that flourish in hydrothermal vent regions only do so because they have specialized organs (Table 8.1, entries 10 and 11) that deliver shelter and sulfide to sulfide-oxidizing, autotrophic bacterial endosymbionts – primarily in exchange for carbon. In the shallow photic zones of tropical oceans, photosynthetic algae (dinoflagellates of the genus *Symbiodinium*) play the role for coral-forming animals that sulfide autotrophs do for hydrothermal vent animals. Carbon fixed by coral-inhabiting algae (at a density as high as 10^{10} algal symbionts per m^2 of coral surface) allows the coral animals (sea anemone-like Anthozoans) to grow and secrete calcium carbonate exoskeletons that accrue and provide crucial habitats for other forms of marine life (entry 12 of Table 8.1). In shallow tropical waters near Hawaii, a squid (*Euprymna scolopes*, Figure 8.6) supports an entirely different type of symbiosis: the exchange of a sheltered habit within the squid's body for an endosymbiotic bacterium's ability to glow (entry 13). The luminescent marine bacterium *Vibrio fischeri* is specifically recognized by the host and is adapted to colonize the squid's light organ, which can eliminate shadows cast by the squid, enabling it to evade nocturnal predatory attack from below.

The next two entries in Table 8.1 (entries 14 and 15) provide additional examples of how the prokaryotic digestive traits can be exploited by animal hosts. On the ocean floor, deceased whale biomass ("whale falls") provide a banquet of resources for heterotrophic life, both microbial and animal. In these whale falls, bone marrow is an unusual resource – rich in cholesterol and collagen and encased in the bone mineral, hydroxyapatite. A worm of the genus *Osedax* (Figure 8.7), adapted to burrow into the bone marrow, relies upon bacterial endosymbionts to digest marrow-derived carbon compounds. Similarly, shipworms (entry 15) bore into submerged wooden materials, but without their nitrogen-fixing, cellulose-degrading bacterial endosymbionts, shipworms would not be successful.

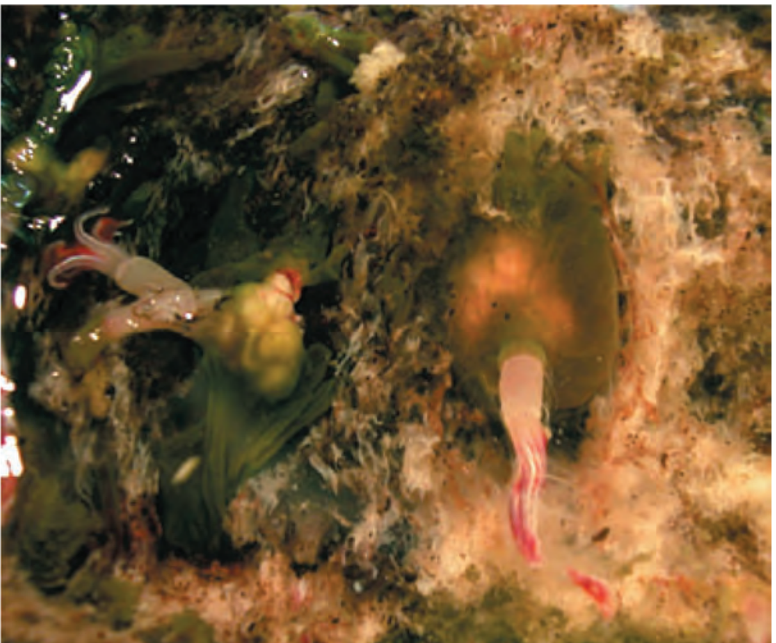


Figure 8.7 Photograph of the bone-eating marine worm, *Osedax frankpressi*, which relies on bacterial endosymbionts for digestion of bone marrow constituents. The image shows two red-and-pink-tufted worms in a whale vertebra with their green roots and white ovisacs exposed. (Courtesy of G. Rouse, Scripps Institute of Oceanography, with permission.)

The penultimate entry in Table 8.1 lists insects as the habitat for symbioses. *Bacteria*-containing "bacteriocyte" structures within insect tissues have been microscopically detected and catalogued for more than four decades (Baumann et al., 2006, 2013; Koga et al., 2012). As many as 10% of some insect families are inhabited by obligate symbiotic bacteria that have not yet been cultivated in the absence of their insect hosts. The relationships between aphids (plant sap-ingesting insects; Figure 8.8) and their endosymbiotic bacteria (*Buchnera*) have been particularly well characterized using nonculture-based molecular procedures, including cloning and sequencing of 16S rRNA genes. The phylogeny of the endosymbiotic aphid bacteria perfectly matches that of the aphid hosts. This provides strong evidence for strict transmission of the endosymbionts from generation to generation of aphid progeny with no influx of genes from other bacteria throughout the 150–250 million years of the evolution of the symbiosis. The implication is that all contemporary aphid–bacterial symbioses are derived from what may have been a single infection by a free-living bacterium long ago. As discussed in Sections 3.2 and 5.9, endosymbionts

Figure 8.8 Photograph of plant sap-ingesting aphids, which harbor the bacterial endosymbiont *Buchnera*. (Courtesy of Wikipedia, GNU Free Documentation License.)

and intracellular parasites are likely to shed genes not required in their new habitats. Indeed, the genome of *Buchnera aphicola* APS (655,725 base pairs (bp)) is one-tenth the size of many free-living bacteria. Physiological, biochemical, developmental, and genetic details of the many types of insect–bacterium relationships (entry 16) await elucidation by ongoing research. The general consensus is that the insect endosymbionts benefit their hosts nutritionally (by synthesizing amino acids and growth factors) and also by conferring resistance to parasites and extending the insect's range of habitats by extending the insect's utilizable food sources.

The final entry in Table 8.1 presents protozoa as habitats that have been colonized by bacteria over evolutionary time. Bacterial endosymbionts are very common in amoebae, flagellates, and ciliates (Figure 8.9). Such associations were detected microscopically more than a century ago. Interest in the bacterial endosymbionts of protozoa is spurred for a variety of reasons that include both evolutionary and medical issues. Because protozoa reside in the lower trunk of the *Eukarya* in the tree of life (see Sections 5.5 and 5.6), our understanding of the development of cellular organelles (e.g., mitochondria, chloroplasts) can be advanced by studying protozoa. Furthermore, there is a continuum of relationships exhibited by intracellular residents of protozoa (from pathogens and parasites to symbionts and organelles) that may provide important insights into the emergence of new

microbial agents of human disease. The physiological impact of many intracellular protozoan residents have not yet been discovered, while others have definitively been shown to benefit their host through mechanisms that include: the synthesis of growth factors (e.g., heme, lysine), serving as an electron sink in the hosts' anaerobic metabolism and conferring resistance to predators.

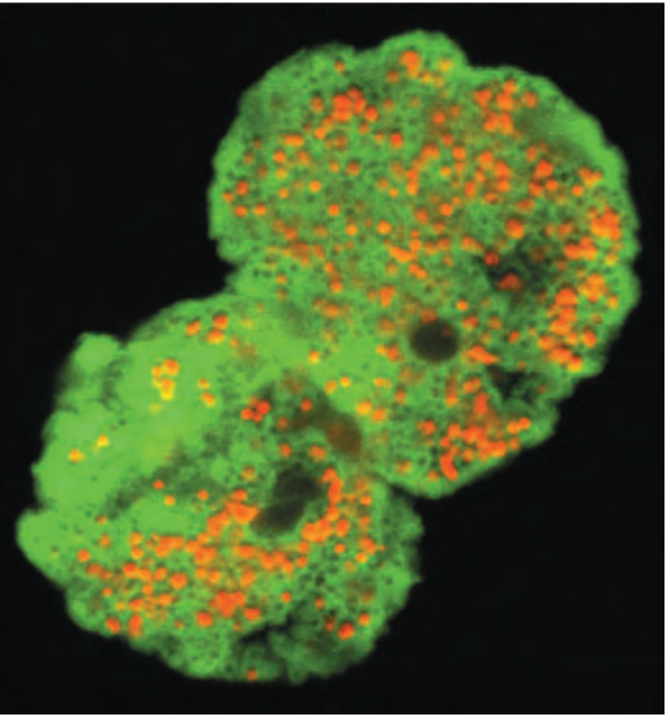


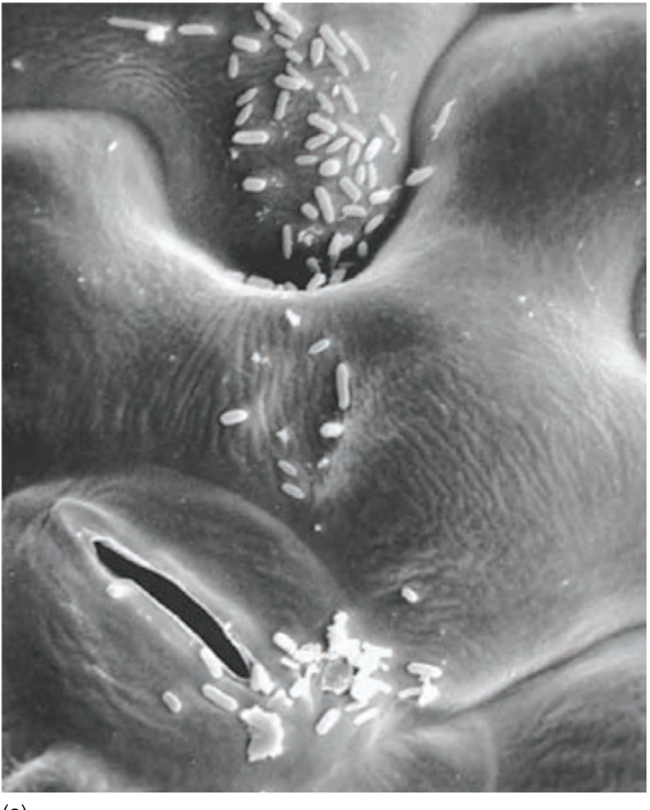
Figure 8.9 Photograph of a protozoan and its intracellular endosymbionts visualized using fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy. The fluorescent probes were designed to specifically target the protozoan host, *Acanthamoeba* (green signals), and the intracellular bacteria, *Protochlamydia amoebophila* (red signals), respectively. Scale of protozoan cell, ~30 µm. (From Horn, M., A. Collingro, S. Schmitz-Esser, et al., 2004. Illuminating the evolutionary history of Chlamydiae. *Science* **304**:728–730. Copyright 2004, AAAS/Science. Courtesy of M. Horn, Universitat Wien, Austria.)

8.2 MICROBIAL RESIDENTS OF PLANTS AND HUMANS

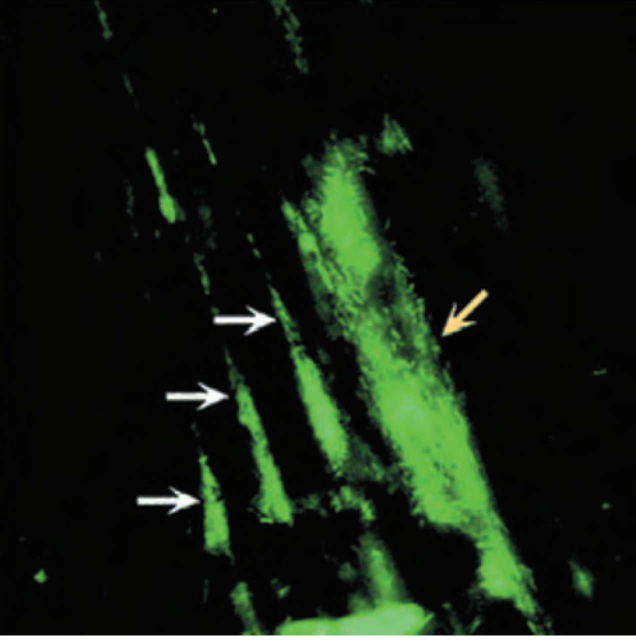
In an insightful early essay entitled, "Eukaryotes as habitats for bacteria", Schlegel and Jannasch (2006) pointed out that "eukaryotes present a multitude of habitats for bacteria . . . the surfaces, cavities, crevices, and intercellular spaces open to the air, as well as intestinal tracts, exudates, and excretory substances offer opportunities for the growth of many bacteria". Earlier in this book, we obtained a preview of how important other life forms can be as microbial habitats: Table 4.9 (Section 4.5) assessed microbial biomass associated with gastrointestinal tracts of animals. Here we briefly survey plants and humans as habitats for microorganisms.

Plants

Plants consist of two primary microbial habitats: the phyllosphere and the rhizosphere (Figure 8.10). The phyllosphere is defined as the aerial portions of plants (trunk, branches, stems, buds, flowers, leaves); phyllosphere inhabitants are termed epiphytes. The majority of information about microbial epiphytes has focused upon the leaf habitat where bacterial densities often average 10^6–10^7 per cm^2 (Lindow and Brandl, 2003; Vorholt, 2012). Given the extensive standing global plant biomass (see Sections 4.1 and 7.6), planetary phyllosphere bacterial populations are impressive – having been estimated at ~10^{26} cells (Lindow and Brandl, 2003; Vorholt, 2012).



(a)



(b)

Thus, the microbial phyllosphere has the potential to influence many processes globally – as well as individual plants locally.

Like all other microbial habitats, understanding the phyllosphere requires that we pose several basic questions:

1 What conditions and resources prevail in the phyllosphere?
2 What organisms occur?
3 What processes occur?
4 What is the motivation for microbiological inquiry?

These four questions are addressed by information presented in Table 8.2. Vorholt (2012) has prepared a recent review of the current state of knowledge of phyllosphere microbiology, including results of "omics"-based investigations, views on cell–cell interactions, and the application of phyllosphere knowledge to crop protection, phytoremediation, and curbing food-borne human pathogens.

Figure 8.10 (a) Microscopic image of microorganisms found on the leaves of a bean plant. (b) Biofilm of fluorescently labeled bacteria on the root of a tomato plant. A large microcolony of bacteria is apparent on the root surface and is indicated by the yellow arrow. The white arrows highlight three smaller colonies that have formed at plant root cell boundaries, which may be the site of release of root exudates used by bacteria as nutrient sources. ((a) Courtesy of G.A. Beattie, Iowa State University, with permission. (b) From Davey, M.E. and G.A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Molec. Biol. Rev*. **64**:847–867. With permission from the American Society for Microbiology.)

Table 8.2
Four fundamental ecological questions and answers about the plant phyllosphere. (Compiled from Lindow and Brandl, 2003; Vorholt, 2012)

Question	Answer
What conditions and resources prevail in the phyllosphere?	• Physical conditions: fluctuations in temperature, humidity, free-standing rainwater, ultraviolet light • Plant microhabitats: veins, stomata, trichomes (leaf appendages), water-exuding pores (hydathodes) • Plant surfaces: waxy surface cuticle, subcuticle voids, intercellular spaces within plant stomata • Carbon sources: waxes, sloughed cells, sugars (glucose, fructose, sucrose), isoprenes, methanol • Spatially heterogenous (microscale) distributions of nutrients (sugars) and micronutrients, such as iron
What organisms occur?	• Patchy, spatially heterogenous distributions of heterotrophic microorganisms and plant pathogens, localized in aggregates of >1000 cells • High community complexity [especially of *Bacteria* (alpha-, beta-, gamma-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*) and fungi; with very few *Archaea*] based on small subunit rRNA gene- and metagenomic sequencing • Methylotrophic organisms enriched by plant release of methanol
What processes occur?	• Commensalistic relationships between plant and epiphytic microorganisms • Competition among heterotrophs for limited nutrients • Cell–cell microbial signaling, that may include quorum sensing molecules and "antibiotics" • Plant immune-like defenses against pathogenic bacteria include: secondary metabolites with antimicrobial activity and localized cell death (the hypersensitive response, which partitions uninfected from infected tissue) • Plant pathogens may release virulence factors that assist in their colonization and infection • Microorganisms may also release surfactants and auxins that assist them colonizing the plant
What is the motivation for microbiological inquiry?	• Understanding the ecology of an important biosphere habitat • To use that understanding to control microbial agents of plant disease, food-transmitted human disease, and crop damage • Microbial ecological strategies can establish microorganisms that are antagonistic to plant pathogens (biological control) and to food-borne human pathogens • Microbial ecological strategies can use competitive exclusion to: (i) curtail establishment by plant pathogens and (ii) curtail frost damage instigated by phyllosphere bacteria that catalyze ice nucleation

Table 8.3

Key types of influence and processes that occur in the plant rhizosphere. (Compiled from Pinton et al., 2001a; Uren, 2001; Mendes et al., 2013)

Type of influence from root or microbe	Examples
Physical change of habitat	Growing root surface extending through soil Moisture flux toward root
Chemical change of habitat	Nutrient (e.g., N, P, K) depletion near root surface Root products: • diffusates (sugars, amino acids, organic acids, inorganic ions, oxygen, growth factors, water) • excretions: CO_2, protons, bicarbonate, ethylene • secretions: mucilage, enzymes, iron-binding siderophores, allelochemicals that inhibit other organisms • debris: root cap cells, sloughed tissues
Microbiological processes in habitat	Mycorrhizal infection Nitrogen fixation Pathogen infection Competition for nutrients among heterotrophs Interactions with soil-fauna (e.g., nematodes, insects)
Plant processes in habitat	Acquisition of nutrients (e.g., by iron uptake or phosphorus solubilization) Acquisition of water via transpiration and as modulated by mucilage release Protection against toxic agents (e.g., complexation of Al^{3+}) Protection against competition and plant pathogens (e.g., allelochemicals that inhibit other organisms) Establishment of symbiotic relationships (root exudates may guide chemotaxis by *Rhizobia* and mycorrhizae)

The term “rhizosphere” was first used by Hiltner (1904) to indicate the zone of soil where exudates released from plant roots have the potential to influence soil microorganisms (Jones, 1998; Bowen and Rovira, 1999; Pinton et al., 2001a, 2001b; Bais et al., 2006; Cardon and Whitbeck, 2007; Mendes et al., 2013). The physical, chemical, and microbiological complexity of the soil habitat have been discussed at length in Sections 4.2, 4.6, 5.1, 5.4, 5.10, 6.10, and 6.11. Soil contains a yet-to-be understood assortment of ~10^9 microorganisms per gram, composed of tens of thousands of species (estimates go as high as 1 million; Mendes et al., 2013). The extension and growth of a root within the soil provides plant-tissue surfaces for microbial colonization and creates microscale biogeochemical gradients that are far different from those in bulk soil (Table 8.3). Because soil is a carbon-limited habitat, it is conceptually obvious that the portion of soil colonized by plant roots (composed largely of metabolically active, growing, leaky, sometimes senescing, carbonaceous tissue) would harbor

microbial populations distinctive from those in bulk soil. The following are crucial for developing an understanding of rhizosphere ecology, microbiology, and biochemistry:

- Knowing the identity of materials released by the plants
- Knowing the constellation of microorganisms present in the rhizosphere (e.g., *Bacteria*, *Archaea*, fungi, nematodes, protozoa, algae, viruses, arthropods)
- Knowing how the members of the rhizosphere system interact (biochemically, ecologically, developmentally)
- Knowing the impact of released materials upon the above interactions

According to Uren (2001), "root products" are all of the substances produced by roots that are released into the rhizosphere (Table 8.3). All variables (e.g., plant type, soil type, plant stresses) aside, approximately 50% of the fixed carbon in photosynthate is committed to roots – half of this is retained as root tissue (25%) and the remainder is relegated to respiration (~15%), debris (~10%), diffusates (~1%; see Table 8.3 for definition), and secretions (~1%). Within Table 8.3 is a summary of the possible impacts and functional roles of root secretions for plants. In assessing the intricacies of rhizosphere processes, it is important to be aware that most compounds do not persist in soil in the free and active form for very long.

> A secretion must be free to diffuse through a portion of the rhizosphere, but a sort of tyranny of distance exists. The longer it takes or the further it must travel, the greater is the chance that it will be rendered ineffective by microbial degradation/assimilation, chemical degradation/reaction, sorption, or a combination of these processes (Uren, 2001).

In Section 6.10 (Table 6.9), we reviewed insights that were gained when the state-of-the-art bar code-based sequencing of small subunit rRNA genes was applied to both field- and laboratory-grown *Arabidopsis* plants. Based on thousands of 16S rRNA sequences, Bulgarelli et al. (2012) contrasted the compositions of microbial communities in three different root zones and concluded that the plant provides major cues that specifically enrich for certain classes of *Bacteria*. The implications are that, over evolutionary time, plants and their beneficial root-associated microorganism have adapted to one another. This message of co-evolution between plant roots and their microbial neighbors is a theme reinforced by metagenomic, metatranscriptomic, and metaproteomic studies (see Section 6.10) completed to date on a variety of model rhizosphere systems (reviewed by Mendes et al., 2013). Clearly, rhizosphere biology and microbiology offer fascinating challenges for future research.

Humans

Humans present several habitats for microbial colonization. Together these habitats support the "human microbiome", which consists of 10^{13}–10^{14} microorganisms. While each prokaryotic cell is small, the tally of

microbial residents amounts to ~10 times the number of human cells (somatic plus germ cells) in the human body. Thus, we humans are "walking minorities" – outnumbered by the (largely) prokaryotic cells that we support externally, and, especially, internally (see below). We are born 100% human but live and die 90% microbial. If we count the number of genes, the human contribution to the total (~22,000 genes in the human genome) is even smaller – amounting to ~1% of the aggregate gene pool (Gill et al., 2006). Here we focus upon the skin and gastrointestinal tract of humans.

Skin

Fundamental ecological characteristics of the human skin and its microbial inhabitants are presented in Table 8.4. The epidermal layer of our bodies serves as a protective barrier that is replaced every 28 days. The outer-surface stratum corneum consists of 25–30 layers of flattened, dead keratinocyte cells that are continually shed by friction and are replaced by cells formed in deeper layers. Spaces between the keratinocytes are filled with epidermally produced proteins, lipids, and fatty acids. Together, the cells and filler serve as a barrier for moisture loss and against entry of foreign matter (allergens, microorganisms, chemical irritants).

Table 8.4

Four fundamental ecological questions and answers about the human skin. (Compiled from Roth and James, 1988; Taylor et al., 2003; Brüggemann et al., 2004; http://www.nuskin.com/corp/science/skinscience/skin_anatomy.shtml; Grice and Segre, 2011; Human Microbiome Project, 2012)

Question	Answer
What conditions and resources prevail in the skin habitat?	• Physical conditions: fluctuations in ventilation, desiccation, light, temperatures, humidity, pH, and releases from glands (pores, sweat, sebaceous (oil production associated with hair follicles), lymph). Prevailing acidic conditions and cool temperatures • *Epidermis*: the outermost surface (~1 mm thick): – 50 to 100 layers of cells that (from top to bottom) include: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale – key cell types are keratinocytes and melanocytes – key biomolecules are keratin and lipids – renewed every 28 days • *Dermis*: located between the epidermis and hypodermis: – fibrous network ~2 mm thick – cell types include fibroblasts, mast cells, blood vessels, and lymph vessels – key biomolecules are collagen (major protein), elastin protein, and glycoaminoglycan "ground" substances • *Hypodermis*: the deepest layer and composed largely of fat cells (for insulation)

Table 8.4 *Continued*

Question	Answer
What microorganisms?	• Both cultivation and metagenomic (non-cultivation-based) procedures have been applied • Two ecological types: stable residents (autochthonous) and transient residents (allochthonous) • Dominant cultivated taxa include *Staphylococcus*, *Propionibacterium*, coryneforms, *Micrococcus*, and yeasts • Dominant taxa from molecular (16S rRNA gene) surveys vary with specific sites (from forehead to nostril to belly button to forearm to back to buttock to heal) and seem to cluster based on common features of moisture regime and density of sebaceous glands • Dominant taxa at the phylum level (from 16S rRNA sequencing surveys; Grice and Segre, 2011) include *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*; by whole metagenomic sequencing (Human Microbiome Project, 2012); the dominant phyla are *Actinobacteria*, *Firmicutes*, *Fusobacteria*, and others • Analysis of 18S rRNA gene sequences has shown that the vast majority of fungal organisms residing on the healthy skin resemble *Malassezia* species • *Demodex* mites are part of the normal skin microflora, residing in the sebaceous glands and hair follicles of the facial skin and increasing in prevalence with age • *Propionibacterium acnes*, a normal skin inhabitant, carries several immunogenic factors thought to trigger acne skin disease
What processes occur?	• Commensalistic relationships between host and dermal inhabitants • In the underarm area (axilla) and other occluded sites, microbial metabolism of skin secretions is thought to create volatile, odorous products • Constant shedding of surface cells and keratin by host • Sebaceous glands, at the base of hair follicles, release oils and lipids that can be metabolized by microorganisms to fatty acids. At pH 5.5, these fatty acids have an antimicrobial effect on many microorganisms • If the epidermis is breached, pathogenic infections may develop. Bacterial pathogens include *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, *Micrococcus*. Fungal pathogens include *Tinea versicolor*, *Tinea pedis*, and *Candida* • Immune-system monitoring and modulation of skin commensal populations. Keratinocyte cells in the skin continuously sample microbial residents (via pattern recognition receptors), which can lead to the release of antimicrobial compounds (cytokines, chemokines, antimicrobial peptides)
What is the motivation for inquiry?	• Medical treatment of dermatological disease • Commercial development of deodorant products • Ecological aspects of disease and disease transmission

Across the geography of the human body, skin conditions vary markedly in pH, moisture, density of hair follicles, gland (sweat, sebaceous, lymph) secretions, temperature, ventilation, and exposure to light (Table 8.4). When ecological conditions are altered (by covering an exposed area of skin with a bandage, washing with soap, or applying topical antibiotics), new selective pressures alter the composition of the microbial community. One key trait that distinguishes transient (allochthonous) skin populations from true (autochthonous) residents is adherence. Normal microbial residents and pathogens of the human skin may feature specialized surface structures, termed "adhesins", that facilitate cell attachment to collagen-rich interstitial areas of the skin (Nitsche et al., 2006).

Based on the carbon sources available to skin residents, the major ecological niche is for commensal heterotrophs that are able to grow on proteinaceous keratin and collagen and/or secreted oils, lipids, and/or organic acids (Table 8.4). Historical information on the identity of skin microflora has relied on cultivation-based procedures. Thus, for decades, key model cultivated, skin-inhabiting microorganisms (see Table 8.4) have been yeasts, *Staphylococcus* (a member of the *Firmicutes*), and *Propionibacterium*, coryneforms, and *Micrococcus* (the latter three are members of the *Actinobacteria*). To a large degree, the ecological relevance of these cultivated microorganisms for the skin habitat has been confirmed now that non-culture-based methodologies (16S rRNA gene sequencing and metagenomic studies; Grice and Segre, 2011; Human Microbiome Project, 2012) have shown that the dominant skin microflora include *Actinobacteria* and *Firmicutes* (See Table 8.4).

Shifts in host immunity and breaches in the epidermis can allow commensal organisms to act as pathogens – as is the case for many medically important infections from the bacterial genus *Staphylococcus*. In addition to obvious medical concerns caused by bacteria and fungi (dermatomycoses), the microbial ecology of human skin impacts how humans perceive one another via body odor. It is widely accepted that underarm zones of our bodies (the axilla) accumulate initially odorless natural skin secretions that are converted to volatile odorous products by our indigenous microbial skin populations (Taylor et al., 2003). Understanding the populations and altering their physiological activity have obvious commercial applications in the personal-care industry (Table 8.4). Nearly two decades ago, Roth and James (1988) completed a review article on the microbiology of the human skin whose concluding paragraph still applies today:

> Humans exist in an environment replete with microorganisms, yet only a few of these microorganisms become residents on the skin surface. These resident flora and the skin constitute a complex ecosystem in which organisms adapt to changes in the microenvironment and to coactions among microorganisms. The skin possesses an assortment of protective mechanisms to limit colonization, and the survival of organisms on the surface lies in part in the ability of the organisms to resist these mechanisms. Microbial colonization on the skin adds to the skin's defense against potentially pathogenic organisms.

Although microbes normally live in synergy with their hosts, at times colonization can lead to clinical infection. Common infections consist of superficial infections of the stratum corneum or appendages, which can respond dramatically to therapy but commonly relapse. In rare circumstances these infections can be severe, particularly in immuno-compromised patients or hospitalized patients with indwelling foreign devices.

Gastrointestinal tract

The gastrointestinal (GI) tract of an adult human is a tube about 10 m in length that begins with the mouth and esophagus and ends with the rectum and anus. In between are organs that process the food, absorb nutrients, absorb fluids, and allow microbial colonization of processed materials so that, in the end, fecal material consists primarily of bacterial biomass. The stomach is a temporary storage vessel for ingested material where gastric juices (especially HCl) are added and where churning action converts the contents to chyme. The three segments of the small intestine are the major sites for exposing materials from the stomach to digestive fluids (from the pancreas, liver, and intestinal walls themselves) and for absorbing digested food stuffs via high surface-area villus cells that line the small intestine walls. The absorbed nutrients are delivered to the circulatory system and liver (Figure 8.11). Undigested food residues, highly colonized by microorganisms, enter the large intestine, whose bands of exterior muscles and inner-surface goblet cells dehydrate and absorb nutrients from the gut contents until they are released as feces.

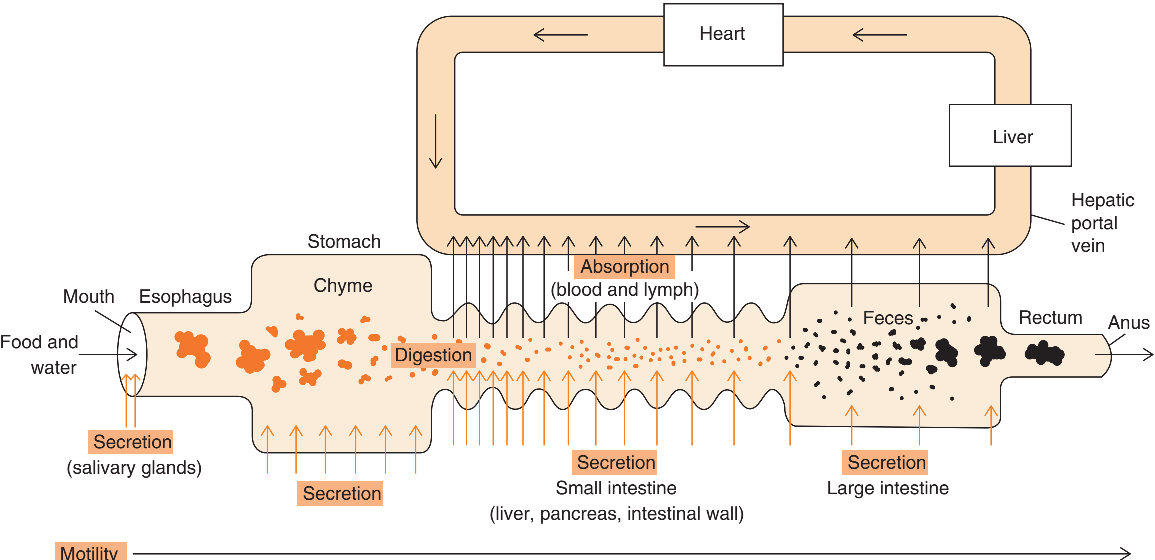


Figure 8.11 Model of the human gastrointestinal tract. (From Vander, A.J., J.H. Sherman, and D.S. Luciano. 1980. *Human Physiology: The Mechanisms of Body Function*, 3rd edn. McGraw-Hill, New York. Reproduced with permission from the McGraw-Hill Companies.)

It is in the large intestine (cecum, rising colon, transverse colon, sigmoid colon, plus descending colon) where a symbiosis (mutualism) analogous to those listed in Table 8.1 (entries 1 and 2, for herbivorous animals) develops for humans (Bäckhed et al., 2005; Walter and Ley, 2011; Grice and Segre, 2012). Figure 8.12 places the gastrointestinal tract (GI) in its ecological and microbiological contexts. The GI habitat (the "inner tube of life": from mouth to stomach, duodenum, jejunem, ileum colon, and rectum; Figure 8.12a) features distinct regimes of both pH and microbial cell density. Cell density in the oral cavity is substantial (10^8–10^9 cells/ml), drops markedly in the stomach, and then gradually rises to reach an extremely high density (10^{11} cells/ml) in the colon. Like all ecosystems, the human GI is constantly immersed in the microbial world. Two categories of microbial populations can be recognized: (i) "autochthonous" natives that are long-term residents who contribute to community function and (ii) "allochthonous" transients that pass through (or perish in) the system, not contributing appreciably to community metabolism or composition. Based on results of both cultivation- and noncultivation-based surveys of the microbial residents, Figure 8.12c depicts a generalized overview of the composition of four key compartments of the GI tract: oral cavity, stomach, small intestine, and large intestine (Walter and Ley, 2011). In Section 6.10, we reviewed detailed methods and results of investigations of the human gut using next-generation barcode-based small subunit rRNA (Table 6.9), metagenomics (Table 6.10), metatranscriptomics (Table 6.11), and metaprotemomics (Table 6.12). Patterns in the resultant data and/or experimental manipulations from many completed investigations address questions that range from "What determines the development of human-gut microbial communities (within an individual, across individuals, within a region, across regions)?" to "What influence does the gut microbiome have upon human health (from digestion to pathology to immunity)?" Answers to these and related questions are intensively being pursued by researchers today (e.g., Human Microbiome Project, 2012; Nicholson et al., 2012; Walter and Ley, 2011; Grice and Segre 2012; Hooper et al., 2012).

Box 8.3 provides a summary of the recognized benefits contributed to humans by their intestinal microflora. Active fermentation of polysaccharides (many not digestible by the human enzymes) leads to microbial production of short-chain fatty acids (e.g., acetate, propionate) that decrease colonic pH, inhibit the growth of pathogens, and provide energy to the colonic epithelial cells, among other things). Also, an intact gut microbiome can shield against the development of a variety of diseases including: inflammatory bowel disease, colon cancer, irritable bowel syndrome, gastric ulcers, nonalcoholic fatty liver disease, and obesity. Finally, the significance of the human gut microbiome is readily appreciated by noting its coevolution with the human immune system, which needs to protect the host from pathogens while simultaneously allowing complex internal microbial communities to flourish because of their key metabolic benefits.

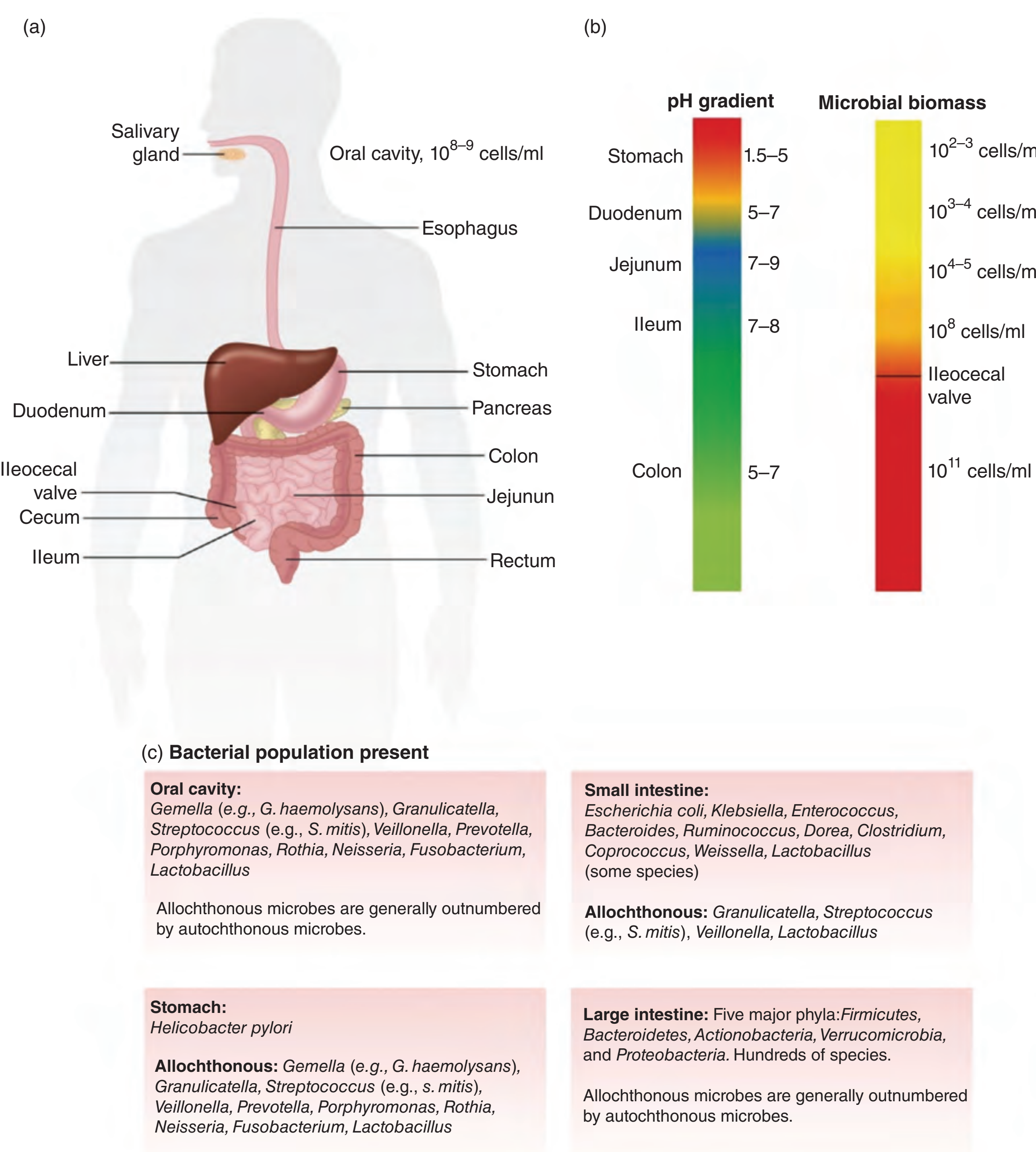


Figure 8.12 Characteristics of the major human gastrointestinal tract and their inhabitants. (a) The major sections of the gastrointestinal tract. (b) Bars in the center indicate pH level moving from stomach to the distal gut (left) and corresponding biomass levels (right). (c) Boxes indicate the dominant types of microbes either allochthonous or autochthonous in those habitats; see text for explanation. (From Walter, J. and Ley, R. 2011. The human gut microbiome: ecology and recent evolutionary changes. *Annu. Rev.* Microbiol. **65**:411–429. Reproduced with permission from Annual Reviews.)

Box 8.3

Recently recognized roles of the intestinal bacterial symbiosis (mutualism) in human physiology, development, and disease. (Compiled from Bäckhed et al., 2005; Eckburg et al., 2005; Gill et al., 2006; Walter and Ley, 2011; Nicholson et al., 2012; Hooper et al., 2012)

Human benefits from intestinal microorganisms

- The distal human intestine is an anaerobic bioreactor programmed with an enormous population (10^{13} to 10^{14} cells).
- Resident microorganisms protect against injury of the human epithelial cells that line the gut.
- The resident microbial community synthesizes and releases nutrients (amino acids and vitamins) used by the human host.
- The microbial community regulates epithelial development within the gastrointestinal tract.
- The microorganisms digest and process (ferment) otherwise indigestible plant polysaccharides, providing the host with ~10% of his/her daily energy needs.
- Differences in microbial community composition among healthy individuals may contribute to differences in individual human physiology and difference in susceptibility to disease.

The gut microbiota in development and disease (Nicholson et al., 2012)

- The influence of the gut microbiota on human health is continuous from birth to old age. The maternal microbiota may influence both the intrauterine environment and the postnatal health of the fetus. At birth, about 100 microbial species populate the colon.
- Early environmental factors (e.g., method of delivery), nutritional factors (e.g., breast or bottle feeding), and epigenetic factors have been implicated in the development of a healthy gut and its microbial symbionts.
- Changes in gut microbial composition in early life can influence risk for developing disease later in life. During suckling, the microbial community develops rapidly, shifts in microbial diversity occur throughout childhood and adult life, and in old age there is a decrease in the *Bacteroidetes* and an increase in *Firmicutes* species.
- The gut microbiota is important for maintaining normal physiology and energy production throughout life. Body temperature regulation, reproduction, and tissue growth are energy-dependent processes that may rely in part on gut microbial energy production.
- Extrinsic environmental factors (such as antibiotic use, diet, stress, disease, and injury) and the mammalian host genome continually influence the diversity and function of the gut microbiota with implications for human health.
- Disruption of the gut microbiota (dysbiosis) can lead to a variety of different diseases, including (A) inflammatory bowel disease, colon cancer, and irritable bowel syndrome; (B) gastric ulcers, nonalcoholic fatty liver disease, and obesity and metabolic syndrome; (C) asthma, atopy, and hypertension; and (D) mood and behavior shifts through hormone signaling.

- The evolution of the vertebrate immune system has been driven by the need to protect the host from pathogens and to foster complex microbial communities for their beneficial effects.
- The gut microbiota is also important for drug metabolism and for preventing the establishment of pathogenic microbes.

The gut microbiota and immunity (Hooper et al., 2012)
- Opportunistic invasion of host tissue by resident bacteria has serious health consequences, including inflammation and sepsis.
- The immune system has evolved adaptations that work together to contain the microbiota and preserve the symbiotic relationship between host and microbiota.
- The evolution of the vertebrate immune system has therefore been driven by the need to protect the host from pathogens that constantly flow through the GI tract and to foster complex microbial communities for their metabolic benefits.

8.3 BIODEGRADATION AND BIOREMEDIATION

This section is designed to familiarize the reader with definitions, principles, and facts about how microbial processes can be used to eliminate environmental pollution. We draw upon fundamentals of physiology (see Chapters 3 and 7) and apply them to technologies aimed at cleaning up both organic and inorganic environmental contaminants.

Background on environmental pollution, biodegradation, and biotransformation

There are a wide variety of industrial organic and inorganic compounds that humans manufacture, use, control, and mismanage. Many of these materials are environmental pollutants, intentionally or inadvertently released to soil, sediment, or aquatic habitats. Examples of environmental pollutants include fossil fuels such as gasoline that contains benzene, toluene, and ethylbenzenes, xylenes (BTEX), and metals such as chromium and mercury, used in industry and commerce.

Obviously, we need to understand the environmental fate of released pollutants because they may threaten human health, ecosystem function, and/or environmental quality. Documenting the environmental fate of pollutant materials is a challenge. Not only is a mass balance-type accounting of pollutants difficult in open field sites, but many competing biotic and abiotic processes may influence pollutant behavior (see Sections 1.4 and 6.3 to 6.8).

The term *biodegradation* is largely synonymous with “microbial metabolism of *organic compounds*”. By breaking carbon–carbon bonds in organic pollutant molecules, microorganisms are often uniquely capable of

altering the chemical structure of the compounds – often rendering them harmless. For a glossary of terms pertinent to biodegradation and bioremediation, see Box 8.4. Biodegradation processes are governed by the fundamental thermodynamic and biochemical principles already discussed in Chapter 3 (Sections 3.6 to 3.11) and applied to the biogeochemical cycling of carbon, sulfur, nitrogen, and other compounds in Chapter 7 (Sections 7.3 to 7.5). Biodegradation reactions are merely the subset of physiological reactions that act upon unusual, often synthetic, industrial organic compounds that are considered environmental pollutants.

Many *inorganic chemicals* (e.g., heavy metals, acid mine drainage, cyanide, mercury, radionuclides, and oxyanions including nitrate and perchlorate) are environmental pollutants whose chemical behavior (especially toxicity, solubility, and mobility) can be altered by microbial processes (see below). The goal of bioremediation technology is to manage biodegradation (organic materials) and biotransformation (both organic and inorganic materials) processes so that environmental pollution is attenuated or destroyed.

Environmental microbiologists frequently use *model systems* to gain physiological, biochemical, and genetic information about biodegradation and biotransformation reactions. Such information is routinely obtained by applying chemical, physiological, and genetic assays to laboratory incubations of flasks containing pure cultures of microorganisms, mixed

Box 8.4

Glossary of terms pertinent to biodegradation and bioremediation. (Reprinted and modified with permission from Madsen, E.L. 1991. Determining *in situ* biodegradation: facts and challenges. *Environ. Sci. Technol.* 25:1662–1673. Copyright 1991, American Chemical Society)

Abiotic reactions. These include all of the reactions not encompassed by biotic reactions. Included are inorganic, organic, photolytic, surface-catalzyed, sorptive, and transport processes.

Biodegradation. A subset of biotransformation that causes simplification of an organic compound's structure by breaking intramolecular bonds. The simplification may be subtle, involving merely a substituent functional group, or severe, resulting in mineralization.

Bioremediation. A managed or spontaneous process in which biological, especially microbiological, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination (*biorestoration* is a synonym).

Biotic reactions. For the purposes of this book, biotic reactions are synonymous with biotransformation reactions. However, depending on the system of interest, biotic reactions may include uptake and metabolism of organic compounds by plants and animals.

Biotransformation. A broad term signifying alteration of the molecular structure of a chemical by microbiological, usually enzymatic, catalysis. Alterations include those that increase (e.g., condensation reactions) and decrease (e.g., mineralization reactions) the number or complexity of intramolecular bonds. Reactions may be both intracellular and extracellular. Biotransformation of both organic and inorganic compounds may be catalyzed by microorganisms – especially oxidation/reduction reactions that may alter the mobility of inorganic compounds.

Cometabolism. Cometabolism is the fortuitous modification of one molecule by an enzyme that routinely acts on another (primary substrate) molecule. The primary substrate supports growth of microorganisms that produce one or more enzymes of low specificity that also act on the cometabolized substrate. The cometabolized substrate is usually altered only slightly and does not enter catabolic and anabolic pathways of the microbial cell. Therefore, the responsible organism does not benefit from cometabolic reactions. Microbial growth does not result and cometabolic reactions are not expected to accelerate. However, other organisms may be able to mineralize products of cometabolism.

In situ biodegradation. In Latin, "in situ" means "in its original place". In situ biodegradation focuses on activating microbial processes for the destruction of environmental pollutants where they are found in the landscape. Mobilizing the pollutants away from the spill site for physical, chemical, or biological treatment in a reaction vessel (i.e., incinerator or bioreactor) is contrary to the definition of in situ biodegradation.

Metabolic adaptation. Metabolic adaptation is operationally defined as an enhancement of biodegradation potential that follows exposure of a microbial community to the organic compounds of interest. The specific mechanisms responsible for metabolic adaptation are seldom investigated, but they include enzyme induction, growth of biodegrading microorganisms, and genetic change.

Microbial metabolism. This term has many commonalities with "biotransformation"; however, the emphasis is upon an integration of physiological pathways and energy flow within an organism. Microbial metabolism is usually proven and explored using laboratory assays that contrast the alteration of organic compounds in the presence of live versus killed microbial cells. Metabolic pathways are discovered by identifying the sequential production of intermediary metabolites (and, ideally, the responsible enzymes and genes that encode those enzymes) produced as microorganisms act upon the compounds.

Mineralization. Conversion of an organic molecule into low molecular weight inorganic constituents (e.g., CO_2, NO_3^-, SO_4^{2-}, PO_4^{3-}) Mineralization occurs when an organic compound is altered by central catabolic cellular mechanisms. The responsible organism(s) typically benefit from mineralization – thus, microbial growth is expected and a substantial portion (~50%) of the carbon in the original organic molecule is usually incorporated into biomass.

Natural attenuation. Generally refers to the physical, chemical, or biological processes which, under favorable conditions, lead to the reduction of mass, toxicity, mobility, volume, or concentration of organic contaminants in soil, sediment, and/or groundwater. The reduction takes place as a result of processes such as biological or chemical degradation, sorption, and others.

cultures, or environmental samples (soil, water, sediment). Under controlled experimental conditions, an "abiotic control" treatment can be prepared that mimics a "live" treatment containing viable microorganisms. Contrasts in the behavior of the environmental pollutant under scrutiny are often crystal clear when comparing live and abiotic treatments: the pollutant is consumed only in the presence of viable microorganisms. In this way, the *roles of microorganisms in biodegradation and biotransformation* are readily documented (see Box 8.4).

Relationships between the technology of bioremediation and the physiological processes of biodegradation and biotransformation

Bioremediation is the intentional use of biodegradation and biotransformation processes to eliminate or attenuate environmental pollutants from sites where they have been released. Bioremediation technologies use the physiological potential of microorganisms, as documented most readily in laboratory assays (see above), to eliminate or reduce the concentration of environmental pollutants in field sites to levels that are acceptable to site owners and regulatory agencies that may be involved (Madsen, 1998).

The fundamental divisions in approaches to implementing bioremediation technology are based on two questions: "Where will the contaminants be metabolized?" and "How aggressively will site remediation be approached?" Regarding location, microbial processes destroy, immobilize, or detoxify environmental contaminants in situ, where they are found in the landscape, or ex situ, which requires that contaminants be mobilized into some type of containment vessel (a bioreactor) for treatment (Figure 8.13). Regarding aggressiveness, *intrinsic bioremediation* is passive – it relies on the innate capacity of microorganisms present in field sites to respond to and metabolize the contaminants. Because intrinsic bioremediation occurs in the landscape where both indigenous microorganisms and contaminants reside, this type of

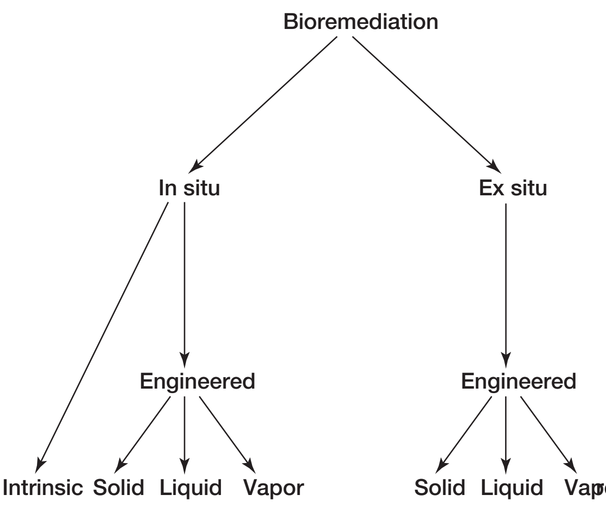


Figure 8.13 Overview of bioremediation approaches. Categories are based, respectively, on where remediation will occur (in situ versus ex situ), on how aggressively remediation is pursued (engineered versus intrinsic) and on the status of the treatment system: solid-, liquid-, or vapor-phase treatments. (From Madsen, E.L. 1998. Theoretical and applied aspects of bioremediation: the influence of microbiological processes on organic compounds in field sites. In: R. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (eds), *Techniques in Microbial Ecology*, pp. 354–407. Oxford University Press, New York. By permission of Oxford University Press, Inc.)

bioremediation necessarily occurs in situ. Alternatively, *engineered bioremediation* takes an active role in modifying a site to encourage and enhance the capabilities of microorganisms to transform and/or biodegrade pollutants. Each of the two major engineered bioremediation approaches may exploit solid-, slurry-, or vapor-phase systems for encouraging microorganisms to proliferate and metabolize the contaminant chemicals (Figure 8.13).

Selection of the most effective bioremediation strategy is based on characteristics of the contaminants (toxicity, molecular structure, solubility, volatility, susceptibility to microbial attack), the contaminated site (geology, hydrology, soil type, climate, and the legal, economic, and political pressures felt by the site owner), and the microbial process that will be exploited, such as pure culture, mixed cultures, and their respective growth conditions, and supplements. Engineered bioremediation relies on a variety of engineering procedures – control of water flow, aeration, chemical amendments, physical mixing, and the like – that influence both microbial populations and targeted contaminants (Madsen, 1998; Atlas and Philp, 2005). Furthermore, the efficacy of the remediation processes must be documented by chemical analysis of water, air, and soil taken from the contaminated site.

Intrinsic bioremediation

Intrinsic bioremediation is the management of contaminant biodegradation without taking any engineering steps to enhance the process. It uses the innate capabilities of naturally occurring microbial communities to metabolize environmental pollutants. The capacity of native microorganisms to carry out intrinsic bioremediation must be documented in laboratory biodegradation tests performed on site-specific samples. Furthermore, the effectiveness of intrinsic bioremediation must be proven with a site monitoring regime that includes chemical analysis of contaminants, final electron acceptors, and other reactants or products indicative of biodegradation processes. This bioremediation strategy differs from the no-action alternative in that it requires adequate assessment of the existing biodegradation rates and potential and adequate monitoring of the process. It may be used alone or in conjunction with other remediation techniques. For intrinsic bioremediation to be effective, the rate of contaminant destruction must be faster than the rate of contaminant migration. These relative rates depend on the type of contaminant, the microbial community, and the hydrogeochemical conditions of the site. Intrinsic bioremediation has been documented for a variety of contaminants and habitats, including low molecular weight polycyclic aromatic compounds in groundwater, gasoline-related compounds in groundwater, crude oil in marine waters (Box 8.5), and low molecular weight chlorinated solvents in groundwater.

Box 8.5

Intrinsic bioremediation of two major marine oil spills and strategies for documenting bioremediation in field sites

I. The *Exxon Valdez* oil spill in Prince William Sound, Alaska

The accident

On March 24, 1989, at 12:03 a.m. the oil tanker *Exxon Valdez* hit Bligh Reef, Prince William Sound, Alaska. Eight of 11 cargo tanks ruptured, spilling 260,000 barrels (41.6 million liters) of heavy crude oil into Prince William Sound. There was immediate exposure of the intertidal and subtidal zones to oil. After 3 days of calm weather and little or no oil-spill-response effort, a major windstorm drove the oil slick southward towards Smith, Naked, and Knight Islands (Figure 1). The oil reached beaches in Chugach National Forest, Kenai Fjords National Park, and other parks and wildlife regions. The oil impacted sites reached as far as 1000 km away and ~2500 km of shoreline was contaminated.

Immediate effects

At the time of the accident, reproduction and migration was in progress for many fish, birds, and mammals. For example, Pacific herring were spawning in intertidal and subtidal eel grass, and juvenile salmon had begun migration towards ocean waters.

The oil contacted and contaminated thousands of seabirds, the salmon hatcheries, the commercial fisheries, and influenced the activities and food sources of many native Alaskans (whose culture is reliant upon fish, shellfish, and wildlife). Salmon egg mortality was high (~67%) for several years. The tally of oil-induced wildlife casualties included 250,000 seabirds, 2800 sea otters, 260 bald eagles,

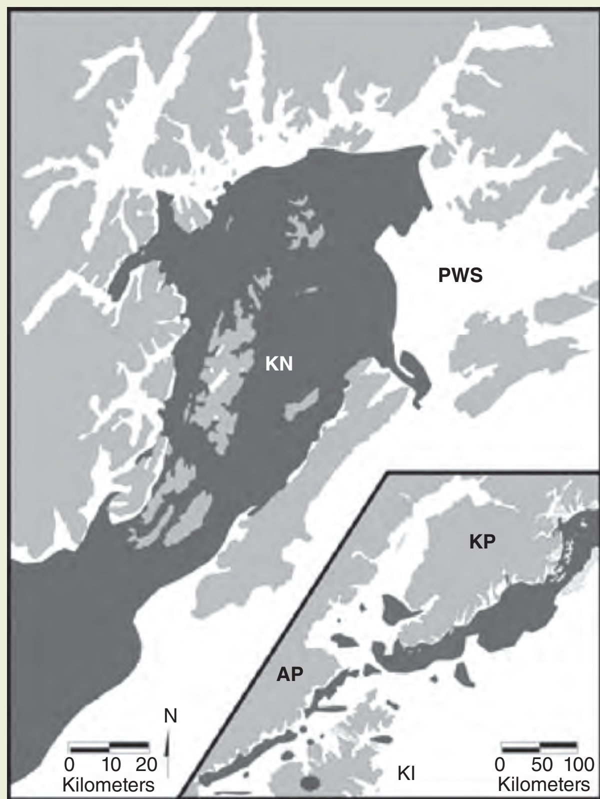


Figure 1 Map of the spread of oil and the shorelines contaminated after the grounding of the *Exxon Valdez* at Bligh Reef in northern Prince William Sound (PWS). Oil was transported to the southwest, striking Knight Island (KN) and other PWS islands, the Kenai Peninsula (KP), the Kodiak Island archipelago (KI), and the Alaska Peninsula (AP). Dark areas are those affected by oil, white areas are open water, and lightly shaded areas are land. (From Petersen, C.H., S.D. Rice, J.W. Short, et al. 2003. Long-term ecosystem response to the Exxon Valdez oil spill. *Science* **302**:2082–2086. Reprinted with permission from AAAS.)

30 harbor seals, and 13 orca whales. The US Fish and Wildlife Service estimated recovery times of 20–70 years for some wildlife populations. Large animals (e.g., Sitka black-tailed deer, brown bear) largely escaped injury.

Cleanup efforts
The initial human, corporate, and governmental response to the spill was largely one of confusion. Organized, coordinated oil recovery efforts were delayed. Opportunities during the first 3 days of calm weather were missed. It was determined that responsibility for cleanup was Exxon's. Cleanup crews were uncertain how to tackle a spill of that magnitude. Technologies were unproven. Booms (used to corral the oil) and skimmers (used to remove oil from the ocean surface) were applied, but the windstorm on day 3 interfered. Chemical dispersants (that diminish the chance of coating the beaches but increase the toxicity of oil) were used to some degree. Due to the windstorm, large masses of oil washed on to coastlines and the oil was redistributed by tides. Attempts were made to "clean" oily shorelines with hot water at high pressure; subsequent evaluations of this approach were that it did more harm (to biota) than good.

II. The BP *Deepwater Horizon* oil spill in the Gulf of Mexico

The accident
On April 20, 2010, high-pressure oil and gas escaped from BP's *Deepwater Horizon* exploratory well in Mississippi Canyon Block 252, 77 km off the shore of Louisiana. Fire and explosions ensued, leading to the death of 11 people. The surface drilling rig burned and sank 2 days later in 1500 m of water. This caused the 53-cm-diameter riser pipe (connecting the rig to the sea floor) to collapse. Oil leaked from multiple locations along the riser pipe. Prior to successfully sealing the leaking well 84 days after the accident, ~4.9×10^6 barrels (779×10^6 liters) of oil had been released (see Figure 2). This oil, classified as "Light Louisiana crude", featured chemical properties (relatively low viscosity and density; higher proportion of low molecular weight compounds) distinctive from the heavy oil released in Prince William Sound, AK. Vast volumes of gaseous hydrocarbons (e.g., methane, ethane, and propane) were also released.

Immediate effects
The spewing gases and oil formed two distinctive plumes in the deep ocean water. Large oil droplets moved upward to the surface to form oil slicks on the water, while neutrally buoyant microdroplets (forming naturally and because of the use of chemical dispersants released intentionally at the seafloor wellhead) formed a "hydrocarbon cloud" of dispersed oil entering currents at a depth of 900 and 1300 m. At the surface, more than 1600 kilometers of Gulf of Mexico shoreline was visibly oiled during the spill. Maximum oiling occurred along the shorelines and barrier islands of Louisiana, Mississippi, Alabama, and western Florida, as well as in the wetland areas of Louisiana (e.g., Barataria Bay). Shoreline cleaning and natural processes have removed a substantial amount of the oil from these areas, leaving a limited number of areas with cleanup still in progress (Barron, 2012).

Reported wildlife oiling during the DWH spill included observations of oiled birds, sea turtles, and dolphins. There were nearly 10,000 observations of oil-impacted birds, including 2085 categorized as visibly oiled alive and 2303 visibly oiled dead. Oil impacts on marine mammals included 140 dead animals (many of them turtles) and numerous dolphins.

Box 8.5 *Continued*

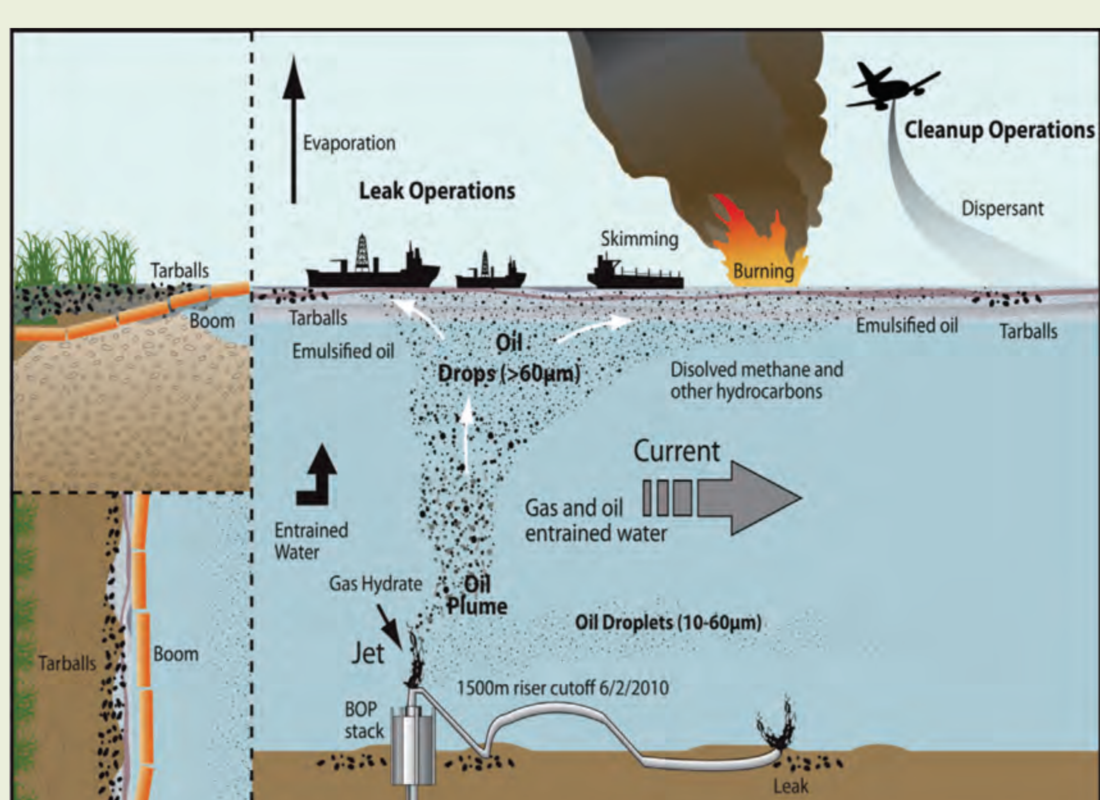


Figure 2 Representation of the *Deepwater Horizon* oil spill site, showing vertical cross-section of the ocean water column, oil dispersal plumes, and cleanup operations on the ocean surface and on shore. (From Terry C. Hazen, Department of Civil and Environmental Engineering, University of Tennessee, with permission.)

Cleanup efforts

Some surface oil slicks were ignited (burned) intentionally, while others were skimmed (to collect the oil), and others received aerially applied dispersant. In total, nearly 7 million liters of chemical dispersants were used; for the first time oil dispersants were released at depth at the wellhead. Large-scale sampling, monitoring, and assessment of subsea, onshore, and offshore water and sediment data were directed by the Operational Science Advisory Team (OSAT) to guide postspill oil removal.

An elaborate series of field- and laboratory-based measurements of geochemical, microbiological, and molecular biomarker (16S rRNA microarray, functional gene microarray, clone libraries, qPCR, phospholipid fatty acids) indicated an extensive response of the indigenous microbial communities to the released gases (methane, ethane, propane) and higher molecular weight hydrocarbons (Hazen et al., 2010; Valentine et al., 2010; Kessler et al., 2011; Atlas and Hazen, 2011). At depth, microbial respiration within both the “hydrocarbon cloud” and the plume of released gases consumed oxygen but never resulted in anoxic conditions.

III. Documenting intrinsic bioremediation in field sites

Intrinsic bioremediation (also termed natural attenuation) is the evolutionarily proven suite of processes that occur due to innate forces of nature (physical, chemical, and biological processes). Many populations of microorganisms native to ocean water possess enzymatic capabilities that allow them to utilize petroleum as a carbon and energy source. These naturally occurring marine microorganisms digest and grow on hydrocarbons. Thus, microorganisms were the key players in destroying the oil released by the *Exxon Valdez* tanker and by the *Deepwater Horizon* oil well. Because ambient levels of nitrogen and phosphorus in ocean water are low, these essential inorganic nutrients limit the physiological activity of the hydrocarbon-degrading microorganisms as they digest large masses of oil. To meet the demand for nitrogen and phosphorus, the cleanup workers in Alaska proposed adding fertilizer to the oil-impacted sites. This fertilization strategy was approved by a US Environmental Protection Agency (USEPA) science advisory board (Table 1).

Documenting bioremediation

- **In open, heterogeneous, dynamic habitats like Prince William Sound and the Gulf of Mexico, how do you prove that the biodegradation processes you hope are occurring truly are occurring?**

Answer: Information in Table 1 provides four examples of strategies that environmental microbiologists have developed for establishing field evidence of biodegradation.

The overarching principle addressed by the strategies in Table 1 is that microbial processes leave a "footprint" in the field that can be documented by drawing on environmental microbiology toolbox (see Sections 5.2 and 6.5).

Table 1

Strategies used to prove that biodegradation by microorganisms in field sites contribute to remediation of organic environmental contamination (Jeon and Madsen, 2013; Bombach et al., 2010)

Strategies for proving field biodegradation	Principles and examples
Replicated field plots with and without applied fertilizer	Some field sites are amenable to controlled treatments designed to stimulate microbiological activity. Comparing the loss of pollutants from plots with and without nutrients can effectively demonstrate the role of microorganisms in field biodegradation. An oil-based (oleophilic) N- and P-fertilizer that adhered to crude oil was dispersed to blocks of the Prince William Sound shoreline impacted by the oil. Oil disappeared more rapidly in treated, compared to untreated, blocks
Internal conservative tracers to document relative disappearance	The loss of certain compounds can be assessed relative to the persistence of less biodegradable, but similarly transported, compounds. Researchers analyzing water-borne hydrocarbons from the *Exxon Valdez* oil spill found that using ratios of straight- to branched-chain alkanes (C_{17}/pristane, C_{18}/phytane), and ratios of other compounds to hopanes in crude oil diminished over time
Molecular biological indicators can qualitatively verify that a suspected process is active	Based on prior knowledge of the biochemical pathways, metabolites, enzymes, and genes responsible for pollutant metabolism, a variety of assays can be applied to field samples. The goal is to find biodegradation-specific biomarkers (e.g., metabolites, mRNA) inside but not outside the contaminated habitat

Box 8.5 *Continued*

Strategies for proving field biodegradation	Principles and examples
Compound-specific isotope-ratio mass spectrometry (CSIA), applied to residual pools of site contaminants in	During biodegradation, pollutant molecules with light isotopic signatures (^{12}C, not ^{13}C and ^{2}H, not ^{3}H) are preferentially metabolized. This leads to an enrichment of ^{12}C in the released CO_2 and an enrichment of ^{13}C and ^{3}H in the residual pollutants left on site. The resulting shifts in ratios of ^{13}C /^{12}C and ^{3}H/ ^{2}H in contaminant pools can be measured by CSIA and attributed to situ microbial activity.

IV. Effectiveness of intrinsic bioremediation in Prince William Sound

There have been several surveys of Prince William Sound aimed at assessing ecosystem recovery. Outstanding questions address: (i) the status of the wildlife and (ii) the petroleum residues remaining in the habitat.

1. Regarding wildlife, experts have concluded that current practices for assessing ecological risks of oil in the oceans should be improved to accommodate site-specific, food web-based toxicology. This approach is required to understand and ultimately predict chronic, delayed, and indirect long-term risks and impacts (Petersen et al., 2003).
2. Regarding petroleum residues, a 2006 survey of 32 shorelines for oil on the land surface and at 0.5 m depth (Short et al., 2004, 2006) revealed that oil remained in 59 of 662 quadrats – largely in the subsurface zones. Persistent oil continues to be documented (Venosa et al., 2010; Li and Boufadel, 2010).

Thus, low-level chronic exposure of marine wildlife continues.

- **If bioremediation is effective, why does oil still persist in Prince William Sound?**

Answer: Intrinsic bioremediation and natural attenuation have successfully removed vast quantities of oil in Prince William Sound. However, some habitats within the sound (especially the subtidal zone) offer suboptimal physiological conditions for oil-degrading microorganisms. The timeframes for nature and microorganisms need not match the impatient expectations of humans.

- **What has led to the remarkable effectiveness of bioremediation in the Gulf of Mexico?**

Answer: “The natural rapid attenuation of oil in the BP *Deepwater Horizon* spill is due to a number of parameters, for example, type of crude oil, offshore, rapid jetting of the oil into the deep sea, rapid dissolution, and microbial adaptation. The Gulf of Mexico has more natural seeps of oil than any marine area in North America, contributing more than 400,000 barrels of oil a year to the Gulf of Mexico. In the Gulf of Mexico the microbiota are likely to be better adapted to oil because of the natural seeps and offshore drilling than almost anywhere else in the world” (Atlas and Hazen, 2011).

Engineered bioremediation

Engineered bioremediation either accelerates intrinsic bioremediation or replaces it completely through the use of site modification procedures, such as excavation, hydrologic manipulations, and the installation of bioreactors that allow concentrations of nutrients, electron acceptors, or other materials to be managed in a manner than hastens biodegradation reactions. Engineered bioremediation is especially well suited for treating nonvolatile, sparingly soluble contaminants whose properties impede successful treatment by other technologies. Engineered bioremediation may be chosen over intrinsic bioremediation because of considerations of time, cost, and liability. Because engineered bioremediation accelerates biodegradation

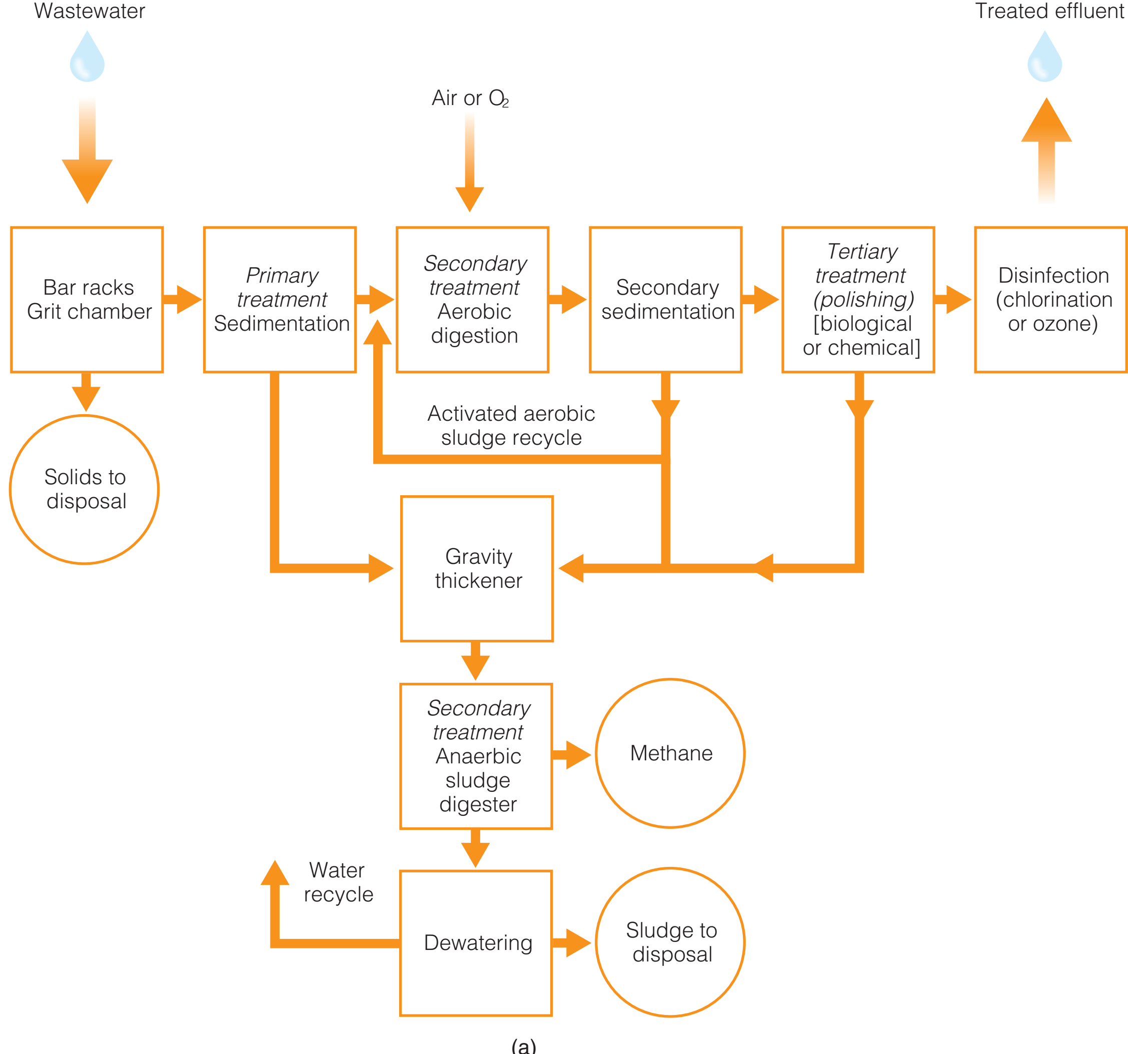


Figure 8.14 (a) Sewage treatment plant design and structure.

(b)

Figure 8.14 (b) City of Ithaca wastewater treatment plant, Ithaca, NY. (Photo courtesy of E. L. Madsen.)

reactions, this technology is appropriate for situations where time constraints for contaminant elimination are short or where transport processes are causing the contaminant plume to advance rapidly. The need for rapid pollutant removal may be driven by an impending property transfer or by the impact of the contamination on the local community. A shortened cleanup time means a correspondingly lower cost of maintaining the site.

Engineered ex situ bioremediation has been used in municipal sewage treatment systems (Figure 8.14) for over a century. In sewage treatment systems, wastewaters from municipalities are directed through an array of controlled environments that encourage microbial growth in filters, tanks, and digestors. Physical, chemical, and microbiological manipulations remove carbonaceous, nitrogenous, and other materials from water before it is discharged into rivers, lakes, or oceans.

The processes that occur in sewage treatment plants (Figure 8.14) are the biogeochemical reactions (especially biodegradation, respiration, nitrification, denitrification, ammonification, sulfate reduction, and methanogenesis) discussed at length in Sections 3.6 to 3.11 and 7.2 to 7.6. In essence, municipal wastewater treatment facilities *intervene* between human activities that load waters with undesirable materials and downstream natural bodies of water. Before the water is discharged from the treatment plant, specialized habitats within the plant create enrichment cultures of mixed, naturally occurring microbial communities. By making a living in these habitats, the

microorganisms degrade organic environmental pollutants, release CO_2, oxidize ammonia, reduce NO_3^- to N_2, generate methane, and accumulate phosphorus. Sewage treatment plants are superb examples of ex situ bioremediation and biogeochemistry. The Sharon–anammox process (Box 8.6)

Box 8.6

Specialized microbiology and engineering for specialized needs: the Sharon–anammox and CANON processes.

I. The Sharon–anammox process

For many years, wastewater engineers have been confronted with a challenging task: "What is the best way to process ammonium-rich sludge-digester effluent?" In standard wastewater treatment schemes, elimination of ammonium requires two steps: (i) the ammonium must be aerobically oxidized to nitrate via nitrification and (ii) this is followed by reduction of nitrate to nitrogen gas via denitrification. A significant amount of oxygen is required for the first step and a significant amount of electron donor (often, added methanol) is required for the second step.

Recently two innovative procedures have been combined to attain a new technology for treating high-ammonium wastewaters. As is clear from the name, the Sharon–anammox process takes advantage of anammox reactions (see Sections 7.4 to 7.5), which, under anaerobic conditions, use ammonium as an electron donor and nitrite as an electron acceptor to produce nitrogen gas.

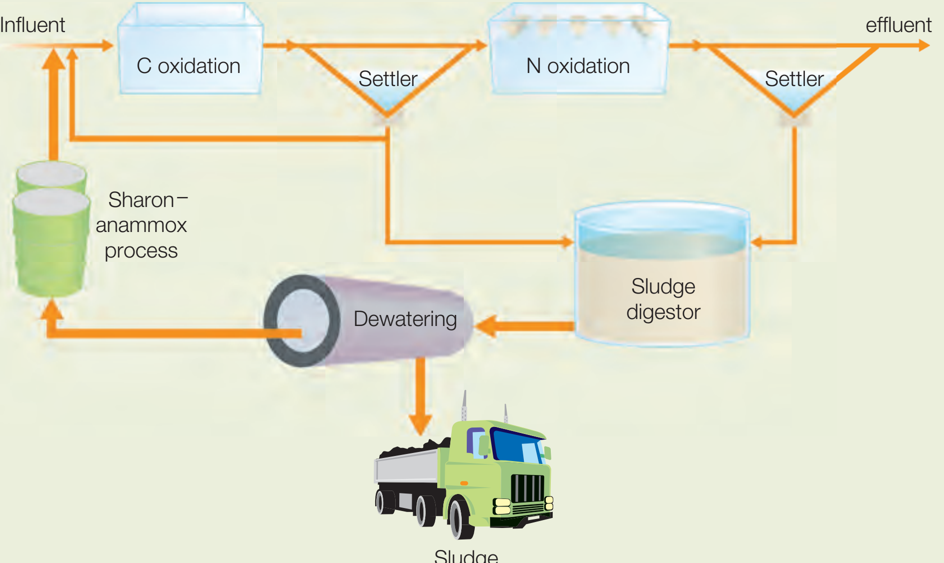


Figure 1 The Sharon–anammox process augments traditional sewage treatment. The high-ammonia effluent from the sludge digestor is freed of nitrogen via the two-step Sharon–anammox process (green tanks). (Reprinted from van Dongen, U., M.S.M. Jetten, and M.C.M. van Loosdrecht. 2001. The Sharon–anammox process for treatment of ammonium rich wastewater. *Water Sci. Technol.* **44**(1):153–160, with permission from the copyright holders, IWA.)

Box 8.6 *Continued*

In the new technology, which is patented and successfully operating on the industrial scale in many European cities, the Sharon process (single reactor system for high-rate ammonium removal over nitrite) prepares the desired 1:1 (nitrite:ammonium) reactant mixture for anammox by delivering a limited amount of oxygen to the high-ammonium waste. High-flow conditions favor rapidly growing *Nitrosomonas* bacteria that convert ammonium to nitrite, while the slower growing nitrite oxidizers are flushed from the system.

A schematic diagram of the Sharon–anammox system in place in the Rotterdam (the Netherlands) wastewater treatment plant is shown in Figure 1. After dewatering, high-ammonium waste from a sludge digestor is delivered first to the Sharon bioreactor (Figure 2) and then to the anammox bioreactor. Nitrogen is effectively removed without any requirement of carbon amendments used routinely in denitrification-based treatment of ammonium. Furthermore, the overall oxygen requirement for nitrogen removal is reduced by 60% and CO_2 emissions drop.

Figure 2 The Sharon (single reactor system for high-rate ammonium removal over nitrite) system bioreactor, Rotterdam, the Netherlands. (Photo courtesy of Grontmij NV, the Netherlands.)

II. CANON

The CANON system (completely autotrophic nitrogen removal over nitrite; also known as one-stage anammox) relies on the same microbiological populations and coupled reactions as the Sharon-anammox process (above; Third et al., 2001; Hu et al., 2013). However, in CANON,

the processes can remove ammonium from wastewater in a single, oxygen-limited treatment step. One-stage wastewater treatment processes have generally lower capital costs than two-stage ones, since no additional tank for nitrite production is required. For the one-stage (CANON) processes, a variety of engineered bioreactor configurations have been used to establish the microaerobic conditions where aerobic ammonia-oxidizing bacteria (AOB) and the anammox bacteria coexist. When biomass is structured in a biofilm or granule (self-aggregated biofilm), AOB are active only in the exterior aerobic outer layer (due to limited O_2 diffusion), while anammox bacteria are active in the internal anoxic core. Aerobic ammonium oxidation produces a suitable amount of nitrite for anammox metabolism and prevents O_2, which would inhibit the anammox bacteria from fully penetrating the biofilm. The choice between the two-step versus the single-step configurations of bioreactors that dispose of excess N via anammox depends on many factors and is, therefore, highly site- and project-specific.

provides an example of how the combination of specialized microorganisms and engineering can address a specialized industrial treatment need: high-ammonium waste streams.

Engineered in situ bioremediation was implemented by R. Raymond and colleagues to clean up petroleum-contaminated groundwater over three decades ago. In the pioneering version of this technology, a groundwater circulation system was established that enhanced the mixing of contaminants, microbial cells, and nutrients designed to encourage aerobic catabolic reactions. Table 8.5 compares in situ and ex situ approaches to engineered bioremediation. In situ bioremediation approaches strive to engineer the landscape to mimic conditions known to foster biodegradation reactions readily demonstrable in laboratory flasks. Thus, the major barrier to successful in situ engineered bioremediation is the impossibility of fully controlling the variety of intractable processes and heterogeneities characteristic of open field sites. Engineered bioremediation must contend with variability in properties of the site, pollutant chemicals, microorganisms, and regulatory agencies overseeing cleanup efforts. Bioreactors central to ex situ strategies to engineered bioremediation offer better control over biodegradation processes, as pollutant metabolism within the bioreactors can be verified and enhanced.

When compared side by side it is clear that the two engineered bioremediation strategies share certain obstacles, but each also offers different advantages (Table 8.5). If contaminants are strongly sorbed on to soil or sediment solids, the ex situ approach may be less appropriate. In either case, qualitative evidence must be obtained to prove that microbiological processes are responsible for pollutant loss. Ideally, quantitative mass balances should be assembled that confirm stoichiometric relationships between physiological reactants (e.g., electron donors and acceptors) and metabolic endproducts (e.g., CO_2, H_2S, CH_4; National Research Council, 2000). Computer modeling of site-specific biogeochemical reactions is one promising approach for quantifying the proportion of pollutant loss

Table 8.5

Comparison of in situ and ex situ strategies for engineered bioremediation systems. (Modified from Madsen, E.L. 1998. Theoretical and applied aspects of bioremediation: the influence of microbiological processes on organic compounds in field sites. In: R. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (eds), *Techniques in Microbial Ecology*, pp. 354–407. Oxford University Press. New York. By permission of Oxford University Press, Inc.)

Feature	In situ strategy	Ex situ strategy
Location	In the landscape	In a controlled bioreactor
Requirements	Engineer the landscape to resemble a laboratory flask	Move contaminants from landscape to on-site bioreactors
Characteristics	Relatively poor control of biodegradation process	Greater control
Obstacles	Complexities of landscape that may prevent success	Complexities of landscape partially overcome
	Pollutant mixtures	Pollutant mixtures
	Unknown site histories	Unknown site histories
	Mass balances uncertain	Decent bioreactor mass balances
	Biotic versus abiotic processes	Biotic processes defined in bioreactor
	Incompatibility between site characteristics and microbiological processes	Incompatibility of site characteristics and microbiological processes can be overcome
	Production of pollutants by microorganisms	Production of pollutants by microorganisms can be minimized
	How clean is clean?	How clean is clean?

attributable to biotic versus abiotic processes when site remediation technologies are implemented. Additional information on bioremediation is provided by Atlas and Philp (2005) and Chakraborty et al. (2012).

Susceptibility of organic compounds to bioremediation

Organic compounds that are most susceptible to microbial metabolism are naturally occurring, have a simple molecular structure, are water soluble, exhibit no sorptive tendencies, are nontoxic, and serve as a growth substrate for microorganisms. By contrast, compounds that are resistant to microbial metabolism exhibit properties such as complex molecular structure, low water solubility, strong sorptive interactions, toxicity, and/or do not support growth of microorganisms. Table 8.6 provides an overview of the categories of contaminants, the mechanisms of microbe–contaminant interaction, the type of contaminant alteration, and the compounds' overall susceptibility to bioremediation measures. Table 8.6 presents a broad perspective on how chemical and microbiological properties jointly affect prospects for bioremediation. Box 8.7 describes the chemical structures for a selection of common organic environmental contaminants.

Table 8.6

Overview of biodegradation and bioremediation potential for particular classes of environmental contaminants. (Modified from National Research Council. 2000. *Natural Attenuation for Groundwater Remediation*. National Academies Press, Washington, DC. Reprinted with permission from the National Academy Press. Copyright 2000, National Academy of Sciences)

Chemical class	Mechanisms of microbe–contaminant interactions	Type(s) of contaminant alteration	Susceptibility to microbiological transformation*	
			Aerobic	Anaerobic
Organic				
Petroleum hydrocarbons				
Low molecular weight				
BTEX	Carbon and electron-donor source	Mineralized to CO_2	1	2
Gasoline, fuel oil	Carbon and electron-donor source	Mineralized to CO_2	1	2
High molecular weight				
Oils, PAHs	Carbon and electron-donor source	Mineralized to CO_2 or partially degraded	1, 2	2, 4
Creosote	Carbon and electron-donor source	Mineralized to CO_2 or partially degraded	1, 2	2, 4
Oxygenated hydrocarbons				
Low molecular weight	Carbon and electron-donor source	Mineralized to CO_2	1, 2	2
Alcohols, ketones, esters, ethers				
MTBE	Cometabolized; occasionally used as carbon and electron-donor source	Partially degraded, sometimes mineralized to CO_2	2–5	4, 5
Halogenated aliphatics				
Highly chlorinated	Electron acceptor under anaerobic conditions; cometabolized	Partially degraded, dechlorinated	2–5	2–5
Less chlorinated	Electron acceptor under anaerobic conditions; carbon and electron-donor source; cometabolized	Partially degraded, dechlorinated	2–5	2–5

Table 8.6 *Continued*

Chemical class	Mechanisms of microbe–contaminant interactions	Type(s) of contaminant alteration	Susceptibility to microbiological transformation*	
			Aerobic	Anaerobic
Halogenated aromatics				
Highly chlorinated	Electron acceptor under anaerobic conditions; carbon and electron-donor source; cometabolized	Partially degraded, dechlorinated	2–5	2, 3
Less chlorinated	Electron acceptor under anaerobic conditions; carbon and electron-donor source; cometabolized	Partially degraded, mineralized to CO_2	1, 2	2
PCBs				
Highly chlorinated	Electron acceptor under anaerobic conditions	Partially degraded, dechlorinated	4	2, 3
Less chlorinated	Electron acceptor under anaerobic conditions; carbon and electron-donor source	Partially degraded or fully mineralized to CO_2	1, 2	2, 4
Dioxins	Electron acceptor under anaerobic conditions	Partially degraded	4	4
Nitrogen-containing explosives (TNT, RDX)	Cometabolized	Partially degraded; immobilized by precipitation or polymerization	2	2
Inorganic				
Metals				
Copper, nickel, zinc	Sorbs to extracellular polymers and biomass	Immobilized by sorption	2	2
Cadmium, lead	Sorbs to extracellular polymers and biomass	Immobilized by sorption; methylation possible	2	2
Iron, manganese	Electron acceptor under anaerobic conditions; oxidized to form insoluble hydroxides; sorbs to extracellular polymers and biomass	Mobility (solubilization) increased by reduction; immobilized by precipitation and sorption	1	1

Chromium	Enzymatically oxidized or reduced to promote detoxification; cometabolized; sorbs to extracellular polymers and biomass	Immobilized by precipitation	2	2
Mercury	Enzymatically oxidized or reduced or methylated to promote detoxification; sorbs to extracellular polymers and biomass	Volatilized or immobilized by sorption, methylation, and precipitation	2	2
Nonmetals				
Arsenic	Enzymatically oxidized or reduced or methylated; electron acceptor under anaerobic conditions; oxidation of reduced forms linked to microbial growth; sorbs to extracellular polymers and biomass	Volatilized or immobilized by precipitation and sorption	2	2
Selenium	Enzymatically oxidized or reduced and methylated; electron acceptor under anaerobic conditions; cometabolized; sorbs to extracellular polymers and biomass	Volatilized or immobilized by precipitation of elemental Se or sorption	1	2
Oxyanions				
Nitrate	Electron acceptor under anaerobic conditions	Reduced to nontoxic nitrogen	4	1
Perchlorate	Electron acceptor under anaerobic conditions	Reduced to nontoxic chloride ion	4	2, 5
Radionuclides				
Uranium	Electron acceptor under anaerobic conditions; sorbs to extracellular polymers and biomass	Immobilized by precipitation	4	2
Plutonium	Cometabolized; sorbs to extracellular polymers and biomass	Mobility increased by reduction to soluble Pu(III); immobilized by precipitation and sorption	4	2
Technetium	Enzymatically oxidized or reduced; cometabolized; sorbs to extracellular polymers and biomass	Immobilized by precipitation	4	2

BTEX, benzene, toluene, ethylbenzenes, and xylenes; MTBE, methyl *tert*-butyl ether; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; RDX, Royal Dutch Explosive; TNT, trinitrotoluene.

*The numeric entries for each compound class provide a rating of susceptibility to microbial transformation under anaerobic conditions (in the presence of oxygen) and anaerobic conditions (when oxygen is absent): 1, readily mineralized or transformed; 2, degraded or transformed under a narrow range of conditions; 3, metabolized partially when second substrate is present (cometabolized); 4, resistant; 5, insufficient information.

Box 8.7

A selection of widespread organic environmental contaminants and their chemical structures

Compound	Chemical structure
Alcohols	R—OH Hydroxyl group Methanol $H_3C—OH$
Alkanes	Saturated with H atoms, no double bonds Octane $H_3C—(CH_2)_6—CH_3$
Alkenes	Sites of unsaturation, at least one C double bond Ethene 2HC═CH2
Alkynes	Triple-bonded C R—C≡C—R Acetylene HC≡CH
Aromatic hydrocarbons	Hydrocarbons containing benzene rings
Atrazine	NH, N, N, NH, N, Cl
Benzene	Aromatic ring C_6H_6
Chlorobenzene	Cl
Creosote	Oily yellow-to-black substance rich in phenols, PAHs, and cresols. Used as a wood preservative
Cresol(s) (three different isomers)	OH, 1, 2, 3, CH_3
2,4-Dinitrotoluene	O, N, O, N═O, O
Dioxins (e.g., TCDD)	Cl, O, Cl, Cl, O, Cl 2.3.7.8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)

Compound	Structure
Esthers	$R—C(=O)—O—C—R$
Ethers	$R—C—O—C—R$
Ethyl benzene	$(C_6H_5)—CH_2—CH_3$
Ketones	$R—C(=O)—R$
Melamine	HN_2, N, NH_2, N, N, NH_2
MTBE (methyl-tert-butyl ether)	$CH_3—O—C(CH_3)_2—CH_3$
Nitrobenzene	O, N, O
Pentachlorophenol (PCP)	Cl, Cl, HO, Cl, Cl, Cl
Perchloroethene (PCE;)	Cl, Cl, Cl, Cl
Phenol	OH
Polychlorinated biphenyls (there are 209 different PCB molecules; shown at right is 2,3,4,5,6-2′, 3′,4′,5′,6-decachlorobiphenyl)	Cl, Cl, Cl, Cl, Cl, Cl, Cl, Cl, Cl, Cl

Box 8.7 *Continued*

Polycyclic aromatic hydrocarbons (PAHs)

Anthracene, a three-ring PAH

Styrene

RDX (Royal Dutch Explosive)

Toluene

TNT (trinitrotoluene)

Trichloroethene

Xylene(s) (three different isomers)

Xylene (o-shown)

Nomenclature: R, organic moiety; —, terminal methyl group (e.g., $-CH_3$).

Petroleum hydrocarbons are naturally occurring chemicals featuring a variety of molecular weights and functional groups (see Section 7.3). BTEX components of gasoline are widely distributed and have been the focus of substantial biodegradation and bioremediation research. BTEX, the low molecular weight fuels, and structurally simple alcohols and ketones are readily mineralizable aerobically; hence, these compounds have been successfully removed from contaminated sites via established bioremediation procedures (see also Table 8.6 and Section 7.3). One prominent fuel component, notably resistant to biodegradation, is methyl *tert*-butyl ether (MTBE). MTBE has been added to gasoline at up to 15% by volume and features three traits (a stable ether linkage, a tertiary carbon structure, and adenosine triphosphate (ATP) generation at or below cell maintenance demands) that prevent MTBE from readily serving as a carbon and electron source for microbial growth. Consequently, MTBE is often a threat to water quality. When microorganisms possess one of a variety of nonspecific oxygenase enzyme systems, oxygen atoms can be fortuitously inserted into MTBE by a process known as a cometabolism (see Box 8.4). Recently, many BTEX components, traditionally considered only susceptible to aerobic microbial attack, have also been found to be biodegradable under a variety of anaerobic conditions (Table 8.6; see also Section 7.3). The high molecular weight petroleum components and creosotes are only slowly metabolized – partly as a result of their structural complexity, low solubility, and strong sorptive characteristics. Thus, bioremediation techniques for the latter classes of petroleum hydrocarbons listed in Table 8.6 are still emerging.

Though halogenated organic compounds have been found in nature, these are not of commercial significance compared to the synthetic halogenated chemicals listed in Table 8.6. When halogen atoms (chlorine, bromine, fluorine) are introduced into organic molecules, many properties, such as solubility, volatility, density, hydrophobicity, and toxicity change markedly. These changes confer improvements that are valuable for commercial products, but also have serious implications for microbial metabolism. The susceptibility of the chemicals to enzymatic attack is sometimes drastically altered by halogenation and persistent compounds often result.

Halogenated aliphatic compounds are straight-chain hydrocarbons in which varying numbers of hydrogen atoms have been replaced by halogen atoms. Halogenated aliphatics are effective solvents and degreasers that have been widely used in many manufacturing and service industries. Some highly chlorinated representatives of this class, such as tetrachloroethene, are completely resistant to aerobic microbial attack, while being susceptible to anaerobic reductive dehalogenation under methanogenic and other anaerobic conditions (Table 8.6). In fact, recent laboratory and field evidence shows that complete reductive dechlorination from tetrachloroethene to the nontoxic plant growth hormone, ethene, can occur (see also Chapter 3, Science and the citizen box). Furthermore, as the degree of halogenation in aliphatics diminishes, susceptibility to other metabolic reactions increases. For instance, vinyl chloride (a carcinogen

created by reductive halogenation of three of the four chlorines on the tetrachloroethene molecule) can be used as an electron donor by both iron-reducing and aerobic microorganisms that oxidize vinyl chloride to CO_2. Aerobic cometabolism of partially halogenated aliphatics (e.g., trichloroethene) has been tested as a bioremediation strategy: microorganisms are supplied with substrates such as methane, toluene, or phenol, which induce expression of nonspecific oxygenase enzymes that insert oxygen atoms across carbon–carbon bonds. This radically alters the toxicity, stability, and biodegradability of the halogenated ethenes. Thus, one treatment approach for chlorinated aliphatics is to remove chlorine atoms anaerobically (via reductive dehalogenation) and then complete the biodegradation process using aerobic cometabolism. Procedures for bioremediating sites contaminated with chlorinated aliphatic are developing rapidly. Both anaerobic treatment (driven by supplementing field-site waters with an electron source for reductive dechlorination) and aerobic cometabolic treatment (driven by additions of methane or aromatic substrates) have been field tested.

Halogenated aromatics, such as phenoxyacetic acid pesticides, pentachlorophenol, polychlorinated biphenyls (PCBs), and others (Table 8.6), consist of one or more benzene rings, which bear halogens as well as other chemical functional groups (i.e., hydroxyls, carboxyls, etc.). The aromatic benzene nucleus is susceptible to both aerobic and anaerobic metabolism, though the latter occurs relatively slowly (see Section 7.3). Overall, however, the presence of halogen atoms on the aromatic ring, their position, and their interaction with functional groups is what governs biodegradability. A high degree of halogenation may prevent aromatic compounds from being oxidatively (aerobically) metabolized, as is the case for PCBs. However, as discussed above for the aliphatic compounds, the highly halogenated aromatics are subject to anaerobic reductive dehalogenation. As the halogen atoms are replaced by hydrogens, the molecules become susceptible to aerobic attack. Thus a common bioremediation scenario for treating soils, sediments, or water contaminated with halogenated aromatic chemicals is anaerobic dehalogenation followed by aerobic mineralization of the residual compounds. It should be noted, however, that when a proper substituent group accompanies the halogens on the aromatic ring, aerobic metabolism may proceed rapidly, as is the case for pentachlorophenol (see Section 8.5).

Other prominent chlorinated aromatic contaminants are dioxins (e.g., tetrachlorodibenzo-*p*-dioxin, TCDD) and polychlorinated dibenzofurans (PCDF). TCDD and PCDF are potent teratogens and carcinogens whose complex chemical structure and strong sorptive properties render them nearly nonbiodegradable – although dechlorination reactions have been reported (Table 8.6). Microorganisms can also metabolize some nitroaromatic compounds, common components of explosives and pesticides (Section 8.5). Dinitrotoluene, produced during the manufacture of polyurethane foams and explosives, is biodegradable (Spain et al., 2000; Section 8.5).

However, despite decades of study, the explosives, trinitrotoluene (TNT) and Royal Dutch explosive (RDX), have not been shown to serve as readily utilizable carbon or energy sources for microbial growth. Under both aerobic and anaerobic conditions, the nitrate moieties on explosives are reduced to amino groups that can cause the compounds to be toxic, undergo polymerization reactions, and/or strongly sorb on to soil solids. Recent reports have shown that aerobically grown bacteria can use TNT and RDX as a nutritional nitrogen source. Nonetheless, the known microbial transformations of dioxins and nitroaromatics are limited in extent (Table 8.6). Bioremediation technology for these compounds is still emerging.

Susceptibility of inorganic materials, including metals, nonmetals, and radionuclides, to bioremediation

Microbiological processes also affect inorganic environmental contaminants – many of which are listed in the lower portion of Table 8.6. Unlike organic compounds that are often susceptible to partial structural alteration or complete detoxification to carbon dioxide by microorganisms, the majority of inorganic contaminant compounds are subject only to changes in speciation that may influence contaminant mobility. Microorganisms may cause precipitation, volatilization, sorption, and solubilization of inorganic compounds. Mechanistically, these reactions can be the result of direct enzymatic processes such as oxidation, reduction, methylation, or uptake – many of which were discussed in Section 7.1. Reaction mechanisms can also be indirect (nonenzymatic), resulting from microbiological production of metabolites or biomass that can strongly influence the behavior of inorganic contaminants via redox, acid/base, coprecipitation, sorption, and other geochemical means. As is evident in the lower half of Table 8.6, microorganisms influence metals, nonmetals, and radionucleotides in diverse ways. Bioremediation technologies for all of the toxic inorganic materials listed in Table 8.6 except nitrate are still emerging.

One nearly universal means by which microorganisms have been shown to reduce concentrations of inorganic contaminants in water (e.g., Cu, Ni, Zn, Cd, Pb; Table 8.6) is by immobilizing aqueous-phase inorganics in microbial biomass and/or microbial exopolymers. The mechanisms involved range from nonspecific electrostatic sorptive interaction between cationic metals and anionic extracellular polysaccharides to highly specific active transport systems that cause metals to accumulate in high concentration within microbial cells. The utility of these sequestration reactions has been proven primarily in engineered wastewater treatment systems where metal-laden water flows over fixed biofilms, which later can be removed from the treatment system so that the toxic inorganics can be recovered. Biomass-mediated sorption reactions clearly influence the behavior of inorganic contaminants. However, any bioremediation technology based on sorption requires periodic harvesting of biomass, which may not be feasible in some settings (e.g., groundwater).

Many inorganic contaminants, especially metals, become relatively soluble, and hence mobile, at low pH. In contrast to the various bioremediation approaches that rely on immobilization reactions, the opposite (washing inorganic contaminants out of a habitat) can theoretically be achieved by directing low pH waters through contaminated sites. The acidification step can be mediated by a variety of microbial processes, including the oxidation of elemental sulfur.

The often highly abundant nontoxic metals, iron (Fe) and manganese (Mn), exist in reduced and oxidized states. The oxidized states (Fe(III) and Mn(IV)) react chemically to form solid oxyhydroxide precipitates that serve as physiological electron acceptors for anaerobic microbial food chains (see Sections 3.7, 3.8, 3.10, and 7.3). The endproducts of Fe and Mn reduction (Fe(II) and Mn(II)) are relatively soluble and may migrate to aerobic habitats, where reoxidation and precipitation can occur chemically with O_2 or be catalyzed by microorganisms. The behavior of many of the toxic metals discussed below is intimately tied to the microbially mediated cycling of Fe and Mn because the toxic metals may be immobilized (through coprecipitation and sorptive reactions with many Fe and Mn oxides) or solubilized [by being reduced via chemical reactions with Fe(II) and Mn(II)]. Thus, most of the inorganic compounds in Table 8.6 are shown to undergo immobilization reactions via sorption and precipitation.

Chromium (Cr) is a metal whose key oxidation states are Cr(VI) and Cr(III). In aqueous environments Cr(VI) predominates as the mobile and highly toxic anions, chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) Reduced Cr(III) is less toxic and less mobile because it precipitates at pH 5 and above. A variety of both aerobic and anaerobic microorganisms have been shown to enzymatically reduce Cr(VI) to Cr(III), but the physiological reason for this ability has not been adequately investigated. Among the hypotheses explaining the reduction reactions are: survival (i.e., detoxification), cometabolism (i.e., fortuitous enzymatic reactions), and the use of Cr(VI) as a physiological electron acceptor (to date, only equivocal evidence for the latter hypothesis has been obtained). Direct microbial detoxification (reduction) of Cr(VI) is unlikely to be a useful remediation technology in anaerobic habitats because the reduction occurs spontaneously in the presence of sulfide, Fe(II), and some organic compounds. Although microbial production of sulfide, Fe(II), and reduced organic compounds is generally reliable, additional research is required before judging if Cr(VI) reduction has the potential to serve as a useful bioremediation tool (Table 8.6).

Mercury (Hg) is a toxic metal whose predominant forms include mercuric ion (Hg(II)), elemental mercury [Hg(0)], and the biomagnification-prone organic mercury compounds, monomethyl and dimethyl mercury. All transformations of mercury by microorganisms are considered detoxification reactions that are intended to mobilize mercury away from microbial cells (see also Sections 7.1 and 7.3). Most reactions are enzymatic, carried out by both aerobes and anaerobes, and involve the uptake of

Hg(II) followed by its reduction to volatile forms (elemental Hg(0), methyl, and dimethyl mercury; Table 8.6). Hg(II) also forms highly insoluble precipitates with sulfide; thus, one indirect microbial detoxification strategy involves the stimulation of sulfate-reducing microorganisms. Engineered systems that first reduce the mercuric ions and then purge the volatile mercury from water have been designed and implemented (Section 8.6).

In addition to mercury, microorganisms are capable of methylating other metals (Cd, Pb, Sn, Te, Se; Se methylation is discussed in detail below). Additional methylation reactions may occur as a result of nonbiological transmethylation by microbially produced methylated donor compounds such as trimethyl tin. These donors may react with ionic forms of palladium, thorium, platinum, and gold, but the resultant reduced metals may not be chemically stable. The significance of these unusual metal methylation reactions for bioremediation is uncertain.

As mentioned in Chapter 7 (Science and the citizen box), arsenic (As) is a toxic element capable of existing in five different valence states (As(III), As(0), As(II), As(III), and As(V)). Forms of arsenic range from sulfide minerals (e.g., As_2S_3) to elemental arsenic to arsenic acid to arsenite (AsO_2^-) to arsenate (AsO_4^{3-}) to various organic forms that include methylated arsenates and trimethyl arsine. Clearly, the chemical and microbiological reactions of arsenic are complex. Both anionic forms (arsenite and arsenate) are highly soluble and highly toxic – interfering with various enzyme functions and oxidative phosphorylation, respectively. No form of arsenic is nontoxic. Microorganisms transform arsenic for three fundamental physiological reasons: (i) under anaerobic conditions, arsenate (As(V)) can be used as a final electron acceptor; (ii) under aerobic conditions, reduced arsenic (e.g., arsenite) has been shown to serve as an electron donor, generating energy (ATP); and (iii) under both anaerobic and aerobic conditions, arsenic can be detoxified by methylation, oxidation, or reduction mechanisms that mobilize arsenic away from microbial cells. Engineered bioremediation strategies that rely on mobilizing methylated arsenic from water have been implemented. All in situ bioremediation strategies for arsenic cleanup need to contend with both the complexities of arsenic microbial transformations and site-specific geochemical conditions. Arsenic bioremediation technology is still emerging (Table 8.6).

Although selenium (Se) is an important and beneficial micronutrient for plants, animals, humans, and some microorganisms (largely because of selenium's role in some key amino acids), this element can be toxic at greater than trace concentrations. In natural environments, selenium has four predominant inorganic species: Se(VI) (selenate, SeO_4^{2-}), Se(IV) (selenite, SeO_3^{2-}), Se(0) (elemental selenium), and Se(–II) (selenide). Like arsenic, selenium also has many volatile organic forms; these include: dimethyl selenide, dimethyl diselenide, methane selanone, methane selenol, and dimethyl selenyl sulfide. Each of these compounds exhibits its own chemical and biochemical behavior, mobility, and toxicity. The various forms of selenium are transformed by microorganisms according to

their physiological needs and the ambient thermodynamic conditions (see Table 8.6). Reduced inorganic selenium compounds have been shown to be oxidized under aerobic conditions, though not linked to microbial growth. Oxidized selenium (selenate) can serve as a final electron acceptor for anaerobic microorganisms, resulting in production of selenide and/or elemental selenium. Methylation of the various selenium compounds is thought to be a protective detoxification mechanism that mobilizes selenium away from microbial cells. Thus, like arsenic, the environmental fate of selenium is governed by complex interactions between chemical and physiological processes. For instance, anaerobic microbial reduction of selenate and selenite to insoluble elemental selenium represents an important mechanism for immobilizing and removing selenium from aqueous solution. Furthermore, the various volatile, methylated forms of selenium are sufficiently mobile that aerobic deselenification (largely via dimethyl selenide formation) of highly contaminated Californian soils has been demonstrated in field experiments. Given the complex chemical and biological processes that influence the fate of selenium, effective bioremediation practices in the subsurface are still emerging (see Table 8.6).

Oxyanions are water-soluble, negatively charged chemical species in which a central atom is surrounded by oxygen. Nitrate (NO_3^-) is a naturally occurring oxyanion commonly found at low concentrations in soils, sediments, and both surface water and groundwater as a result of the biogeochemical cycling of organic matter (see Sections 7.3 and 7.4). Nitrate is a readily utilizable form of nitrogen that can be assimilated into amino acids by plants and microorganisms. Although serving to supply nitrogen, an essential nutrient, nitrate is also a serious health concern for at least two reasons: (i) it can be a chemical or microbiological precursor for nitrite, which spontaneously combines with amino compounds to form highly carcinogenic nitrosamines, and (ii) nitrate can be reduced to nitrite in the stomachs of infants, which can cause the respiratory stress diseases, methemoglobinemia.

Nitrate is produced from ammonia by nitrifying microorganisms under aerobic conditions. The major microbial process that destroys nitrate is dissimilatory reduction to dinitrogen gas (denitrification; see Sections 7.3 and 7.4). Genetic, biochemical, physiological, ecological, environmental, and engineering aspects of denitrification have been examined for decades. As shown in Table 8.6, nitrate is used as a physiological electron acceptor under oxygen-free (anaerobic) conditions. The denitrification process is widespread among microorganisms and occurs reliably in every anaerobic habitat with abundant carbon and electron sources. Denitrification is a well-established bioremediation process and is used routinely in sewage treatment plants to curb eutrophication.

The oxyanions chlorate (ClO_3^-) and perchlorate (ClO_4^-) or their precursors (chlorine dioxide, hypochlorite, chlorite) are produced by a variety of paper manufacturing, water disinfection, aerospace, and defense industries. Many of these oxygenated chlorine compounds have recently been

found to be naturally occurring at trace concentrations. These highly oxidized forms of chlorine are energetically very favorable physiological electron acceptors for microorganisms. Compared to denitrification, knowledge of chlorate and perchlorate biodegradation reactions is limited. However, laboratory studies using both pure bacterial culture and environmental samples (soil, freshwater sediments, sewage) have shown that in the presence of common electron donors (carbohydrates, carboxylic acids, amino acids, even H_2 and H_2S) microorganisms can grow at the expense of perchlorate and chlorate, thus reducing them to the nontoxic chloride ion. Furthermore, bioreactors have recently been engineered to successfully convert chlorate and perchlorate to chloride. Thus, the prospects for (per) chlorate bioremediation are favorable.

Uranium (U) is a radioactive element that releases α-, β-, and γ-radiation that can be toxic. Uranium can exist in the oxidation states of U(0), U(III), U(IV), U(V), and U(VI), though in nature insoluble U(IV) and soluble U(VI) predominate. U(VI), in the form of the uranate oxygen complex $(UO_2)^{2+}$, has been shown to serve as a terminal electron acceptor for anaerobic microorganisms (see Table 6.14 and Section 6.11); thus, in anaerobic habitats, growth-linked reduction (and hence immobilization) of uranium should be a reliable process (Williams et al., 2012). Although the reverse process, microbial oxidation of U(IV) to U(VI) under aerobic conditions has been demonstrated, this process has not been shown to be physiologically beneficial to the responsible microorganisms. Direct chemical oxidation of U(IV) by molecular oxygen (creating U(VI)) may also influence the robustness of uranium bioremediation strategies. Other radioactive elements, plutonium (Pu) and technecium (Tc), have also been shown to be susceptible to microbial transformation. Iron-reducing microorganisms were found to reduce insoluble Pu^{4+} to the more soluble Pu^{3+}; thus, oxidation/ reduction reactions provide tools for emerging bioremediation strategies for U, Pu, Tc (Table 8.6), and other radionuclides.

8.4 BIOFILMS

Biofilms are matrix-enclosed microbial accretions that adhere to biological or nonbiological surfaces (Hall-Stoodley et al., 2004; Kolter and Greenberg, 2006; McLoon et al., 2011; Berk et al., 2012; Wolcott et al., 2012). Typically, biofilms form in habitats such as submerged surfaces exposed to flowing water (Figure 8.15). However, knowledge of biofilms is also crucial for understanding the microbiology of other habitats, ranging from plant roots to sewage-treatment plants to the human lung and medical devices implanted in the human body. A summary of the major attributes of biofilms is presented in Table 8.7.

The fundamental lesson emerging from the study of biofilm microbial communities is that the suspended (planktonic) lifestyle is very different from the attached lifestyle: the behavior and physiological, biochemical,

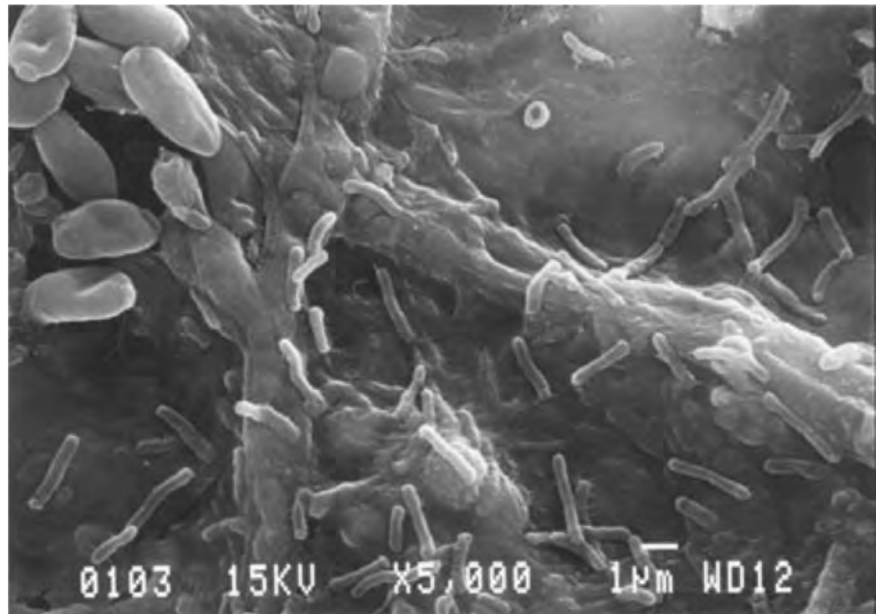


Figure 8.15 Microbial biofilm from the hull of an ocean-going ship. (Courtesy of R. Gubner, Korrosions- och Metallforskningsinstitutet AB, Stockholm, Sweden, with permission.)

and genetic responses of microorganisms in biofilms are distinctive from responses of the same microorganisms when living as isolated suspended cells.

Hall-Stoodley et al. (2004) have pointed out that remnants of 3.2 billion-year-old filamentous biofilms were found in the Kornberg formation in South Africa. The implication is that there have always been strong physiological and ecological advantages for microorganisms in nature to form aggregations of cells attached to solid surfaces. By adhering to one another and to solid surfaces, microorganisms are able to modify their immediate surroundings (their microhabitat) – buffering it against rapid physical and chemical fluctuations (Table 8.7). Such surface associations also take advantage of ionic, electrostatic, and hydrophobic interactions that occur between minerals and many other solid surfaces that tend to concentrate important nutrients in aqueous habitats.

Naturally occurring biofilms may be found in our mouths, in toilet bowls, on submerged aquatic plants, and on medical devices (such as catheters and prosthetic heart valves) (Hall-Stoodley et al., 2004; McLoon et al., 2011; Berk et al., 2012; Wolcott et al., 2012). Biofilm development has also been examined in model systems of culturable bacteria – leading to detailed biochemical and genetic information about events that govern biofilm development. Box 8.8 provides a diagrammatic view of the broad

Table 8.7

Attributes of biofilm microbial communities. (Compiled from Davey and O'Toole, 2000; Hall-Stoodley et al., 2004; Ramey et al., 2004; Kirisits and Parsek, 2006; Kolter and Greenberg, 2006; McLoon et al., 2011; Berk et al., 2012; Wolcott et al., 2012)

Attribute	Details
Biofilm definition	Aggregates of microbial cells associated with solid surfaces
Biofilm composition	Adherent cells in a complex matrix of extracellular polymeric substances (EPSs) including exopolysaccharides, proteins, and DNA
Physical aspects of biofilm formation	At the nanometer and micrometer scale, chemical, nutritional, and physical properties of aqueous solutions change at the solid–liquid interface
Selective pressures for biofilm formation	Resistance to fluctuations in physical/chemical stressors: pH, temperature, light, O_2, hydrodynamic shear stress, dehydration
	Complementary nutritional and physiological associations between bacteria (e.g., anaerobic food webs)
Specialized microbiological aspects of biofilm formation	Cell–cell signaling often known as "quorum sensing" (see Box 8.9) Resistance to doses of chemotherapeutic agents (such as antibiotics) that are lethal to planktonic microorganisms
	Genetic exchange between densely packed microbial cells
	Other density-dependent ecological processes such as viral infections and predation by protozoa

theme of biofilm development and three individual bacterial variations on the theme. The following are the main features of biofilm development:

1. Suspended planktonic cells have specific extracellular surface structures (e.g., flagella) essential for reaching and remaining on a surface.
2. The expression of specific genes (and their protein products) allows attached cells to proliferate, forming a monolayer.
3. Cell proliferation and the expression of additional sets of genes control the formation of microcolonies.
4. A "mature" biofilm often features a network of three-dimensional towers and channels whose formation is, again, linked to particular expressed genes.
5. The life of any particular biofilm is dynamic and subject to hydrodynamic stresses of flowing water. The fragmentation and release of cells back into the planktonic phase occurs during all biofilm developmental phases.

As is clear from details presented in the text of Box 8.8, variations on the five-part theme (above) are species-specific. Intracellular, genetic, and regulatory cues are distinctive for the three pure-culture bacterial systems described in Box 8.8. In the *Pseudomonas aeruginosa* system, development from the microcolony to the mature biofilm stage is dependent upon both the synthesis of alginate (an extracellular polysaccharide that augments the biofilm's intercellular matrix) and a density-dependent cell–cell signaling system known as "quorum sensing" (Box 8.9). Clearly biofilms play a very important role in many naturally occurring, engineered, and medical systems. Biofilm research will continue to be a major frontier in environmental microbiology far into the future.

Box 8.8

Biofilm development. (Compiled from Davey and O'Toole, 2000; Moorthy and Watnick, 2005; Ramsey and Wozniak, 2005; Van Houdt and Michiels, 2005; Kolter and Greenberg, 2006; McLoon et al., 2011; Berk et al., 2012; Wolcott et al., 2012)

Figure 1 outlines the three models for the early stages in biofilm formation in three of the best-studied model organisms, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Vibrio cholerae*.

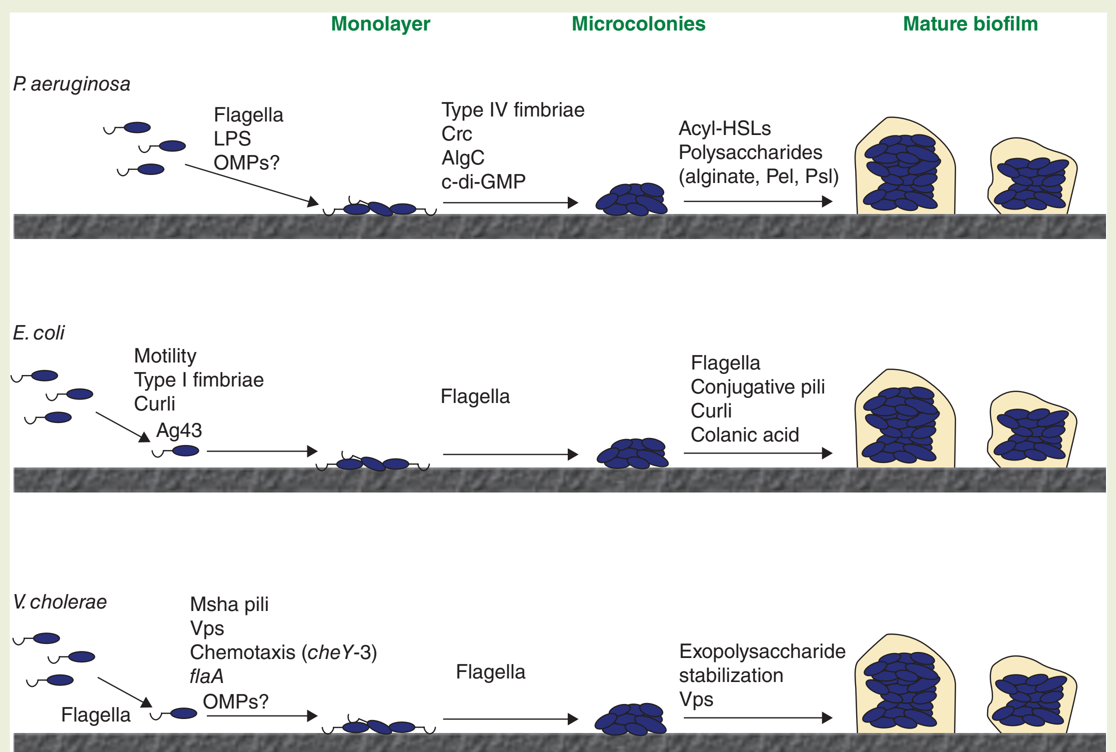


Figure 1 Biofilm development in three Gram-negative organisms. (Modified from Davey, M.E. and G.A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Molec. Biol. Rev*. **64**:847–867. With permission from the American Society for Microbiology.)

In *P. aeruginosa*, flagella are required to bring the bacterium into proximity with a solid surface, and lipopolysaccharide (LPS) mediates early interactions, with an additional possible role for outer membrane proteins (OMPs). Once bacteria are on the surface in a monolayer, type IV fimbriae-mediated twitching motility is required for the cells to aggregate into microcolonies. The production of fimbriae is regulated at least in part by nutritional signals via a protein known as Crc. Documented changes in gene expression at this early stage include the messenger molecule c-di-GMP, upregulation of the polysaccharide biosynthesis genes (alginate, *pel*, and *psl*) and downregulation of flagellar synthesis. The production of cell-to-cell signaling molecules (acyl-HSLs; see Box 8.9) is required for formation of the mature biofilm. Alginate (AlgC) may also play a structural role in this process.

In *E. coli*, flagellum-mediated swimming is required for both approaching and moving across a solid surface. Organism–surface interactions require surface appendages (type I fimbriae and curli) and the outer membrane protein Ag43. Finally, colanic acid, curli, and conjugative pili are required for development of the normal *E. coli* biofilm architecture.

V. cholerae, like *E. coli*, utilizes the flagella to approach and spread across a solid surface. Chemotaxis (CheY-3), Msha pili, and possibly one or more unidentified outer membrane proteins, are required for attachment to the surface and monolayer formation. This initial surface attachment appears to be stabilized by extracellular polysaccharide (EPS). Formation of the mature biofilm, with its associated three-dimensional structure, also requires production and stabilization (via Bap1 and LeuO proteins) of EPS. Vps refers to the EPS produced by *V. cholerae*. The *flaA* gene encodes the flagella subunit protein.

8.5 EVOLUTION OF CATABOLIC PATHWAYS FOR ORGANIC CONTAMINANTS

There are approximately 2×10^7 naturally occurring organic compounds in the biosphere globally and ~100,000 distinct natural products in a given freshwater sediment sample (Seffernick and Wackett, 2001; Wackett and Hershberger, 2001). Furthermore, several million organic compounds have been synthesized by organic chemists (Seffernick and Wackett, 2001). Many synthetic compounds have novel molecular structures (e.g., polymeric plastics and halogenated pesticides like DDT (dichloro-diphenyl trichloroethane)), though the uniqueness of synthetic ("xenobiotic") compounds is not easy to discern, given the immense and unknown diversity of naturally occurring compounds.

Current dogma in the biodegradation literature is that "all naturally occurring organic compounds are biodegradable". A corollary of this is that "truly novel molecular structures should not be biodegradable because there is no evolutionary precedent, no selective pressure, for their metabolism". After all, genetic mutation involves alteration of an existing DNA sequence in a way that varies the catalytic or binding site of a protein.

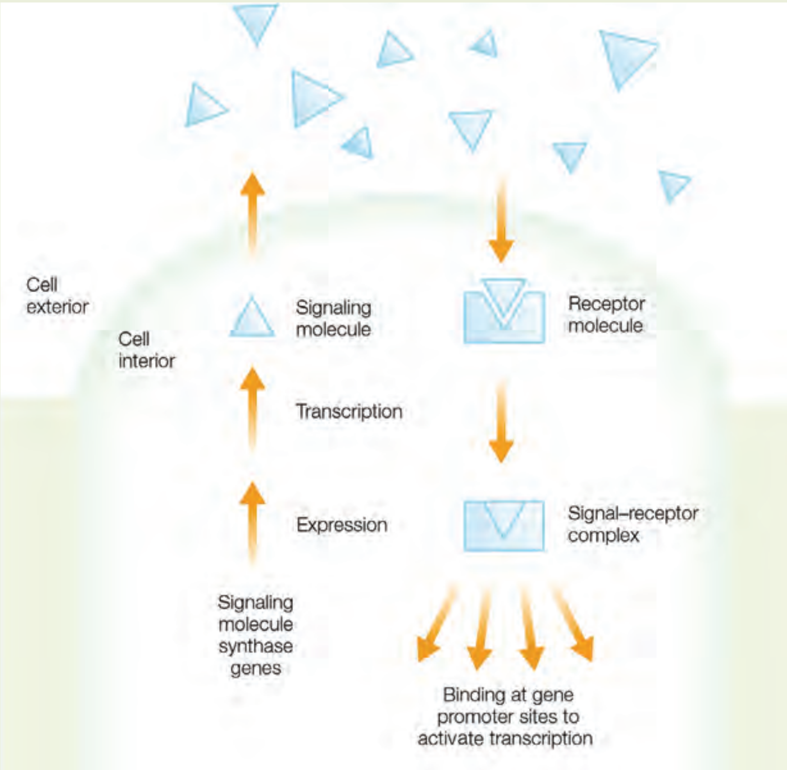
Box 8.9

Quorum sensing is a general cell–cell communication system important for some biofilms and in a variety of other microbial habitats. (Compiled from Miller and Bassler, 2001; Winans and Bassler, 2002; Schuster et al., 2013; Rutherford and Bassler, 2013)

Quorum sensing is a chemical communication system that is widespread in prokaryotes. It is used by bacteria to sense one another and to coordinate gene expression among neighboring microbial populations. In *Pseudomonas aeruginosa*, the small excreted organic molecule, known as acyl-homoserine lactone (acyl-HSL in Box 8.8), has the potential to bind to a specific cellular receptor. Once bound together, the acyl-HSL–receptor complex activates the transcription of genes crucial for mature biofilm development by *P. aeruginosa*. Gene activation (and translation

Box 8.9 *Continued*

of the encoded proteins) will only occur when the concentration of acyl-HSL is above a threshold that is reached when a critical mass of *P. aeruginosa* cells are adjacent to one another. A generalized scheme for quorum-sensing-type regulatory circuits is shown in the figure below.



Quorum sensing has many manifestations in coordinated stimulus response systems of the microbial world (Miller and Bassler, 2001; Winans and Bassler, 2002; Schuster et al., 2013; Rutherford and Bassler, 2013). This cell–cell communication phenomenon was originally discovered in *Vibrio fischeri*, where quorum sensing regulates expression of bioluminescence (Nealson and Hastings, 1979). Quorum sensing is also a regulatory mechanism for coordinating populations of bacteria involved in the following:

- The production of antibiotics.
- The activation of pathogenesis factors.
- Triggering intracellular transfer of plasmid DNA.
- The morphological development of some microorganisms.

A diversity of chemical signaling compounds has been discovered. The compounds are all produced intracellularly, diffuse outside the cells, and only reach the critical intracellular threshold for gene activation when a high density (a "quorum") of cells are present. What emerges is a sophisticated and widespread network of cell–cell signaling system that modulate microbial behavior and interactions in nature.

However, if there is no selective pressure for the genetic change, it is unlikely that a newly evolved protein function will persist and proliferate in the gene pool. Selective pressures are crucial for identifying new changes in protein function that are beneficial to the host.

In the past several decades, we know of a handful of novel synthetic organic compounds that were initially considered nonbiodegradable and later deemed biodegradable (often under restricted physiological conditions; Table 8.8). Among the compounds listed in Table 8.8 are monomers used in pesticides, chemical feed stocks, PCBs, chlorinated solvents, and polymeric products. An extremely insightful source of evidence for the evolution of new biodegradation pathways in microorganisms is the molecular biology and biochemistry of the metabolic pathways themselves

Table 8.8
Selected examples of organic environmental pollutants that were initially considered nonbiodegradable and later reclassified as biodegradable. (From J. Spain, personal communication; Seffernick and Wackett, 2001; Wackett et al., 2002; Johnson and Spain, 2003; Van Der Meer and Sentchilo, 2003).

Compound	Use	Physiological role of compound	Genetic change conferring biodegradability
Melamine	Building block for early industrial polymers	Electron-donor and carbon source	New enzymatic activity, melamine deaminase, in new combination with other genes
Atrazine	Chlorinated triazine herbicide	Electron-donor and carbon source	New enzymatic activity, especially atrazine chlorohydrolase, in new combination with other genes
Styrene	Monomer for industrial polymer, polystyrene	Electron-donor and carbon source	New enzymatic activity, styrene epoxide isomerase, in new combination with other genes
2,4-Dinitrotoluene (2,4-DNT)	Manufacture of polyurethane foam; production of explosives	Electron-donor and carbon source	New enzymatic activity, 2,4-DNT dioxygenase, in new combination with other genes
Chlorobenzene	Solvent for pesticide formulations; degreasing agent	Electron-donor and carbon source	New enzymatic activity, chlorobenzene dioxygenase, in new combination with other genes
Nitrobenzene	Production of the industrial compound, aniline; ingredient in shoe and floor polishes	Electron-donor and carbon source	New enzymatic activity, nitrobenzene nitroreductase, in new combination with other genes
Pentachlorophenol (PCP)	Antifungal wood preservative	Electron-donor and carbon source	New enzymatic activity, PCP 4-monooxygenase, in new combination with other genes

Table 8.8 *Continued*

Compound	Use	Physiological role of compound	Genetic change conferring biodegradability
Polychlorinated biphenyls (PCBs)	Dielectric fluid used in electrical transformers	If highly chlorinated, final electron acceptor If lightly chlorinated, electron-donor and carbon source	(There are 209 different forms (congeners) of PCB molecules; explanations for metabolism resist generalization)
Tetrachloroethene and trichloroethene	Industrial solvents and degreasing agents	Final electron acceptors	New enzymatic activity, tetrachloroethene reductive dehalogenase

Several of the listed compounds (especially styrene) may be naturally occurring. Thus, some metabolic pathways may have evolved prior to widespread manufacture and environmental release of the compounds.

(Johnson and Spain, 2003; Van Der Meer and Sentchilo, 2003). Table 8.9 provides a listing of key types of evidence for recent and ongoing evolution of catabolic pathways for pollutant compounds: patterns in sequenced biodegradation genes, their regulation, and the low specificity of encoded enzymes. The characteristics indicative of recent catabolic pathway evolution are consistent with and predictable from the four proposed mechanistic steps of pathway evolution shown in the lower half of Table 8.9 (Johnson and Spain, 2003).

Concrete examples of the principles of catabolic pathway evolution shown in Table 8.9 are detailed below for the biodegradation of atrazine and chlorobenzene.

Table 8.9

Evidence for the recent evolution of catabolic pathways for pollutant compounds and proposed mechanistic steps that produce new pathways (compiled from Johnson and Spain, 2003)

Biochemical and genetic evidence for early stage of catabolic pathway evolution

- Scattered organization within the host cell of the genes encoding the catabolic enzymes
- Primitive or inefficient regulation of enzyme synthesis
- Poorly adapted degradative enzymes (low affinity for the substrate and low catalytic specificity)
- Remnants of mobile genetic elements (such as transposons) that may have transferred the genes from other hosts

Proposed evolutionary steps that lead to new metabolic pathways

- Gene duplication and mutation allow for alteration or broadening of the substrate preference of the enzymes
- Genes and gene clusters are recruited and assembled via various horizontal-gene transfer mechanisms
- Variant strains of microorganisms able to use the new substrate can proliferate and selective pressure allows strains carrying the best adapted enzymes to thrive
- Further advantage is granted when organisms develop mechanisms that regulate the synthesis and activity of the enzymes

Case studies for the evolution of catabolic pathways: atrazine and chlorobenzene

Microorganisms evolved to be able to utilize atrazine and chlorobenzene as growth substrates. Discovering how this evolution occurred elegantly illustrates environmental microbiological inquiry (Box 8.10). Information in Box 8.10 focuses primarily upon the natural history and genetic basis of atrazine and chlorobenzene metabolism. The early industrial monomer, melamine, is the first entry in Box 8.10 because its preexisting biodegradation pathway set the stage for atrazine.

Box 8.10

Three case studies demonstrating the evolution of metabolic pathways for the biodegradation of organic environmental contaminants. (Compiled from van der Meer et al., 1998; Seffernick and Wackett, 2001; Wackett et al., 2002; Müller et al., 2003)

Compound and its use	Details documenting evolution
Melamine: triazine synthetic polymer intermediate; introduced in the early 1900s	Considered nonbiodegradable in the 1930s, but reclassified as slightly biodegradable in the 1960s. Melamine is metabolized by five enzymes encoded by five genes (*trzABCDE*) to urea, which is hydrolyzed to ammonia and CO_2. The middle intermediary metabolite is cyanuric acid. The gene *trzA* encodes hydrolytic deamination reactions and is a member of the amidohydrolase family of enzymes
Atrazine: major triazine agricultural herbicide used to protect corn; introduced in the 1960s	Considered nonbiodegradable until the mid-1990s when bacteria able to grow on atrazine were isolated. Extensive biochemical and genetic characterization of atrazine biodegradation show that: • the metabolic pathway converts atrazine to cyanuric acid in three steps • the genes coding for enzymes responsible for the three steps (*atzABC*) all belong to the same family of enzymes (amidohydrolase), closely related to *trzA* (for melamine) • nearly identical *atzABC* gene sequences have been found in atrazine- metabolizing bacteria in North America, Europe, Australia, and Asia • although the genes have been found on plasmids (extrachromosomal, often self-transmissible, independently replicating, genetic elements), no single type of plasmid is common to all atrazine-degrading bacteria • sequence analysis of DNA flanking the *atz* genes has discovered evidence for transposons. Thus transposons may be involved in horizontal gene transfer of atrazine biodegradation genes between different hosts

Box 8.10 ***Continued***

Compound and its use	Details documenting evolution
Chlorobenzene: solvent in pesticide formulations, also used as a degreasing agent; produced by chlorination of benzene in the presence of a catalyst; first synthesized in 1851; used in high volume during World War I	Chlorobenzene contamination persisted in a shallow aquifer at Kelly Air Force base for ~30 years. In the 1990s the levels of contamination began to decline Chlorobenzene-degrading bacteria were not in the contaminated zone prior to the chlorobenzene spill, nor could they be isolated outside the contaminated zone. However, bacteria able to grow on toluene and chlorocatechol were present in the indigenous microflora across the site A bacterium able to grow on chlorobenzene, *Ralstonia* sp. JS705, was isolated from the contaminated aquifer and was characterized biochemically and genetically The chlorobenzene degradation pathway and the gene fragments encoding the enzymes represent a merger of two biodegradation pathways: toluene (*todCBA*) and chlorocatechol (*clcAB*). Thus, the chlorobenzene genes in the strain JS705 were assembled in situ within the contaminated aquifer Analysis of the DNA sequence of the new combination of genes allowed the investigators to reconstruct the genetic history of strain JS705. It was originally a toluene-degrading bacterium and received the chlorocatechol gene on a self-transmissible mobile genetic element, termed a "genomic island" (see Chapter 5, Science and the citizen box). Sequence analysis also implicated involvement of insertion (IS) elements, and hence transposons (other agents of gene rearrangement), in achieving the final configuration of genes conferring chlorobenzene metabolism to strain JS705

Atrazine is a member of the s-triazine class of herbicides, developed in the 1950s, that kill susceptible plants by attaching to the quinone-binding protein in photosystem II (Wackett et al., 2002). The atrazine structure is similar to the diazine structures of DNA bases, cytosine and adenine, but the atrazine molecule features three major alterations (a Cl group, a third N within the ring structure, and *N*-alkyl substitutions; see Box 8.7) that impede microbial metabolism. Until the 1990s, all biodegradation studies showed that atrazine could not be used as a carbon and energy source by soil microorganisms: at best, a nonbeneficial (cometabolic) minor change in the atrazine molecule was documented. However, after 1993 several independent investigations were able to detect, isolate, and characterize bacteria that used atrazine as a carbon and energy source for growth. Extensive biochemical and genetic characterization of the *Atz* gene cluster, its related genes and enzymes, and the mobile genetic elements that carry them (Box 8.10) makes it clear that a combination of gene duplication, mutational refinement, recombination, and proliferation was the broad mechanism that made atrazine biodegradable around the globe today.

The last case study in Box 8.10 describes microbial metabolism of chlorobenzene. The scenario portrayed for chlorobenzene is very different from that of atrazine because it is site-specific. Chlorobenzene is a colorless organic liquid

with a faint, almond-like odor whose primary industrial use is the manufacture of dyestuffs and insecticides. It is also a solvent used in adhesives, drugs, rubber, paints, dry cleaning, and textile manufacture. Long-term exposure to chlorobenzene may damage the liver, kidney, and central nervous system of humans. Information presented in Box 8.10 describes how chlorobenzene spilled into the subsurface at Kelly Air Force Base (near San Antonio, Texas), persisted for several decades, and then the concentrations began to decline. Culturable bacteria capable of growing on two compounds related to chlorobenzene (toluene, chlorocatechol) were found throughout the study site. Only after natural attenuation (see Section 8.3) of chlorobenzene had begun, was it possible to isolate (from the contaminated portion of the study site) bacteria that utilized chlorobenzene as a carbon and energy source. Biochemical and genetic analysis of the genes conferring chlorobenzene metabolism on the newly isolated bacterium revealed that the new gene cluster was a hybrid of those from toluene and chlorocatechol metabolic pathways and that two mobile genetic elements (a "genomic island" and transposons) were the likely causes of site-specific evolution of chlorobenzene catabolism (Box 8.10). Juhas et al. (2009) have recently provided an insightful and expanded overview on the role of genomic islands in microbial evolution, including the evolution of catabolic pathways.

8.6 ENVIRONMENTAL BIOTECHNOLOGY: OVERVIEW AND NINE CASE STUDIES

Definitions and scope

According to Glick and Pasternak (2003), the term *biotechnology* was created in 1917 by a Hungarian engineer, Karl Ereky, to describe an integrated process for the large-scale production of pigs using sugar beets as their source of food. In Ereky's view, biotechnology was "all lines of work by which products are produced from raw materials with the aid of living things". In 1961, biotechnology was redefined by Carl Göran, as "the industrial production of goods and services by processes using biological organisms, systems, and processes" (Glick and Pasternak, 2003). A twenty-first century definition of biotechnology might be "the integrated use of biochemistry, molecular, biology, genetics, microbiology, plant and animal science, and chemical engineering to achieve industrial goods and services". An example is production of human insulin by cloning the gene into the bacterium, *Escherichia coli*.

It follows that *environmental biotechnology* is a subset of biotechnology that addresses environmental needs and problems, environmental goods and services. Therefore, what are environmental needs, problems, goods, and services?

Figure 8.16a highlights several of the major global environmental problems faced by humans in the twenty-first century. Figure 8.16b illustrates the intricate connections between biotechnology and environmental biotechnology, between human needs, commercial needs, cultural needs, and between goods and services, economy and ecology.

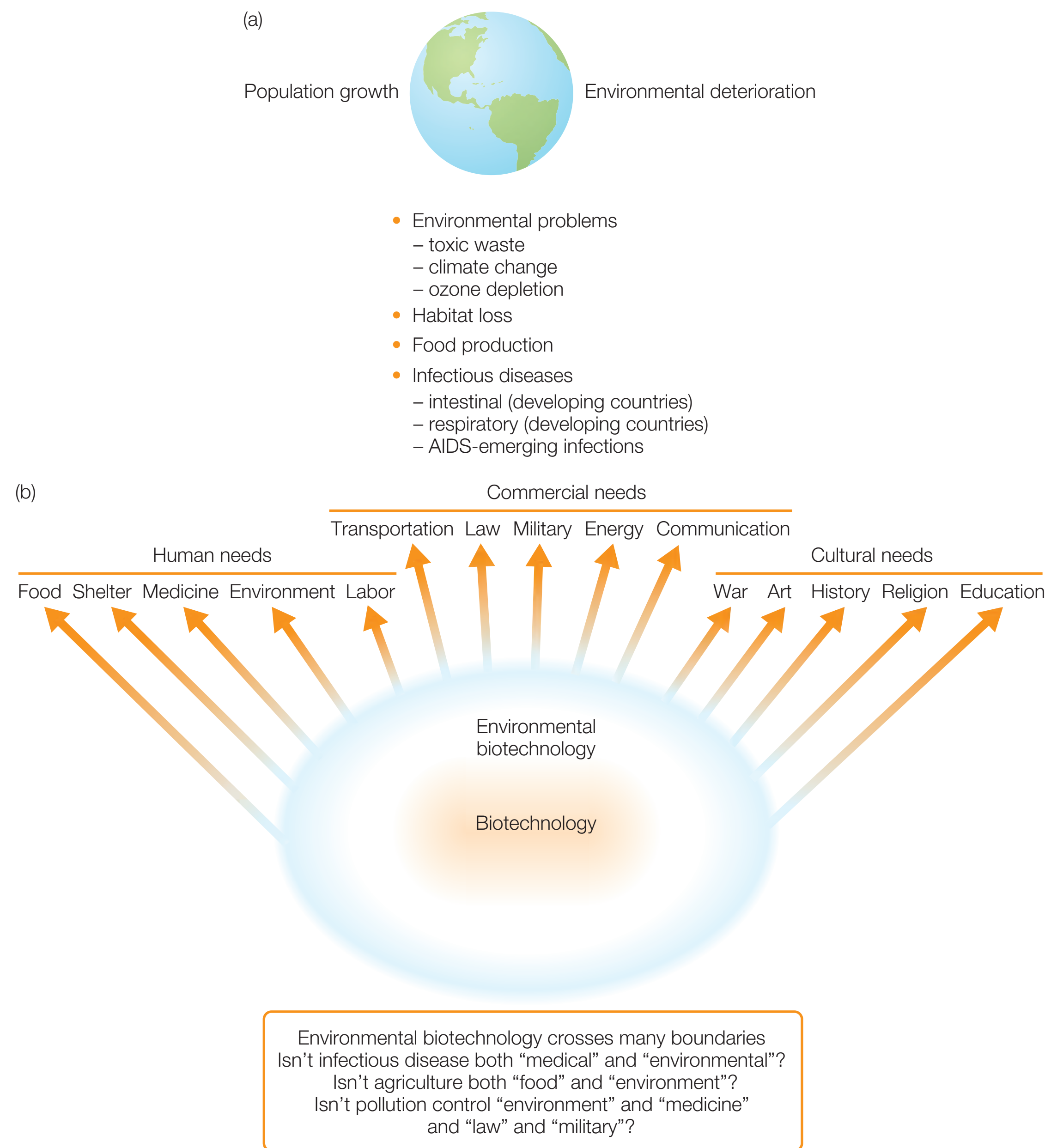


Figure 8.16 Overview of global environmental problems in the twenty-first century and the cross-disciplinary nature of environmental biotechnology. (a) The relationships between human population growth, environmental deterioration, and health. (b) The "goods and services" needed by humans are provided, in a cross-disciplinary manner, by biotechnology and environmental biotechnology.

Many boundaries between disciplines and human endeavors are crossed by environmental biotechnology. It extends from pollution control to food production and to medicine. Throughout this book, we have found that connections are both implicit and explicit in the science and practice of environmental microbiology and biogeochemistry. At the level of an individual bacterial cell, the cycles of carbon, nitrogen, sulfur, iron, and other elements are intimately linked (see Chapters 3 and 7). Similarly, at the levels of global circulation in the atmosphere and the oceans (see Chapter 4), human activities in one place influence (e.g., emissions of CO_2, chlorofluorocarbon refrigerants, or the pesticide, DDT) conditions in other places.

Traditional environmental biotechnology includes: tilling the soil, sowing seeds, harvesting crops, plant breeding, seed storage, *Rhizobium* inoculation for enhanced nitrogen fixation, composting, forestry, aquaculture, wastewater treatment, constructed wetlands, and microbial leaching of mining ores for metal recovery. More recent environmental biotechnology developments have utilized genetic engineering approaches to address goods and services related to environmental and related concerns. The immense breadth of environmental technological issues (e.g., Evans and Furlong, 2011) cannot be treated here. However, nine case studies that sample the issues and approaches to environmental biotechnology appear below.

Our current civilization is fueled largely by petroleum, natural gas, and coal. There is a growing awareness that the economic, political, and environmental costs of fossil fuels are high (Logan, 2004; Chu and Majumdar, 2012). Therefore considerable intellectual and technological efforts are being directed toward discovering renewable energy sources for the twenty-first century. The first three case studies address energy biotechnology. The next four case studies illustrate how genetic engineering of microorganisms and plants has the potential to improve sustainability in agriculture, manufacturing, and environmental cleanup. The final two case studies provide examples of how molecular detective work can discover the origin of human-disease outbreaks: the emergence of severe acute respiratory syndrome (SARS) and epidemic cholera in Haiti.

Background to genetic engineering

Genetic engineering (heralded as the "eighth day of creation", Judson, 1996) has transformed biology from a descriptive science into a powerful technological tool (Glick and Pasternak, 2003; Primrose and Twyman, 2006). In a nutshell, DNA technology allows DNA fragments (genes) to be identified, characterized, modified, and used to create novel products (chemicals or organisms) or processes, or (in the case of gene therapy) to cure diseases by correcting their genetic causes *in vivo*. A major success story in genetic engineering is the production of the human hormone, insulin (critical for treating diabetes). Diabetes affects millions of people who, due to a deficiency in insulin production, cannot regulate their own blood-sugar levels. Prior to genetic engineering, insulin was extracted from the pancreas of cows and pigs. The human genes for insulin were cloned

and expressed in the bacterium *E. coli*. Now genetically engineered insulin is an improvement over cow and pig insulin, plus it is more abundant and less expensive.

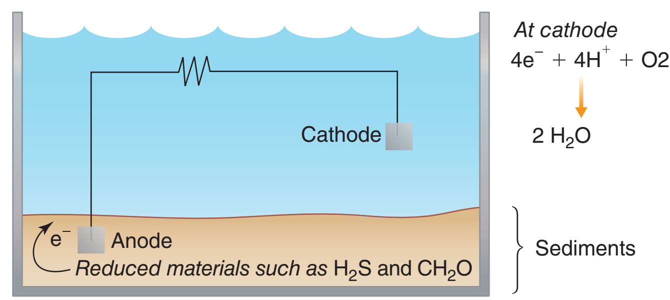
Case study 1: harvesting electricity from anaerobic bacteria – microbial fuel cells

Sections 3.8 to 3.10 emphasized that a major theme of the "anaerobic way of life" is contending with final-electron-acceptor-limited habitats. Electron sinks are critical for ATP generation during electron transport in microorganisms. In essence, electrons in reduced substrates (e.g., carbohydrates, organic acids, H_2, S^{2-}, CH_4) begin an "electric circuit" that ends when the electrons reach the electron sink furnished by a final electron acceptor. In 2002, Bond et al. reasoned that it might be possible to establish an electrical connection between electron-acceptor-limited microorganisms in anaerobic sediments and the best electron sink of all, molecular oxygen (see Section 3.8). An electrode (anode) was inserted into highly reducing marine sediments rich in carbon and active anaerobic microorganisms. The anode was connected in an electric circuit to a cathode positioned in adjacent aerobic (O_2 rich) water. Bond et al. (2002) were able to measure an electric current in the circuit. The milliamp-level electric current was able to power small scientific devices that might be deployed in remote locations (Figure 8.17). Furthermore, the output of electric current from model fuel cell systems could be boosted simply by delivering a pulse of the reduced-carbon electron donor (Figure 8.17). In essence, electricity was generated via oxidation of reduced carbon.

Confirmation and refinement of these anaerobic electric fuel cells was obtained by Chaudhuri and Lovley (2003), who found that a pure culture of the iron-reducing bacterium *Rhodoferax ferroreducens* was able to carry out the same type of electric-current generation described above at ~80% efficiency, fueled with dissolved sugars, including glucose, fructose, sucrose, and xylose. The science and technology of microbial fuel cells has undergone a variety of scientific and technological developments during the ensuing years (witness reviews by Logan and Regan (2006), Logan et al. (2006), Logan (2009), and Logan and Rabaey (2012)). This has spawned a new scientific field of "Microbial Electrochemistry", which focuses on detailed mechanisms by which electrons are transferred outside microbial cells to insoluble (solid) materials that can serve as anodes and/or cathodes in electrical circuits (Lovley and Nevin, 2011; Malvankar et al., 2011) . The term "exoelectrogen" applies to the microorganisms involved in these electron-transfer reactions. Well-known exoelectrogens include *Geobacter* and *Shewanella*. Another key frontier within the discipline of microbial electrochemistry is the role of filamentous microorganisms (and associated nanowires) in establishing "long distance" electric currents within naturally occurring microbial communities. Filamentous "cable bacteria" in marine sediments have been shown to establish electric currents that couple oxygen reduction at the surface of marine sediments to the oxidation of sulfide more than a centimeter below (Nielsen et al., 2010; Schauer et al., 2014).

It is possible that the basic principle of the microbial fuel cell shown in Figure 8.17 can be the basis for specialized, highly engineered electrical generation systems in the future. Logan and Rabaey (2012) have pointed out that domestic, industrial, and animal wastewater together contain ~1.5×10^{11} kilowatt-hour (kW h) of potential energy (~17 GW of power). Capturing part of this energy via microbial fuel cells would provide a new source of electrical power that would also avoid the consumption of energy for wastewater treatment. Another aspect the emerging discipline of microbial electrochemistry is "microbial electrosynthesis", in which

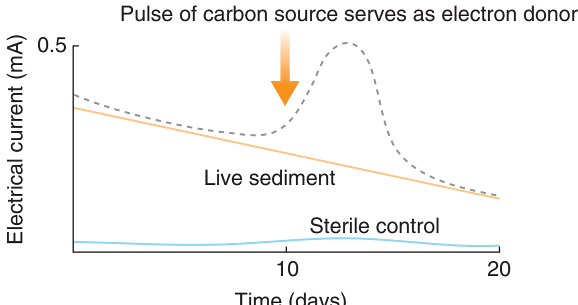
(a) • Harvesting electricity



• = Resistor ≡ Appliance ≡ Voltmeter, ammeter

(b) • Power generation in W/m^2 of electrode surface

Pulse of carbon source serves as electron donor



(c) • *Contemporary application*: Power for marine oceanographic instruments (sensors, hydrophones, etc.)

• *Future application*: Highly engineered, highly efficient microbial fuel cells

Figure 8.17 Generation of electricity by microbial physiological processes in sediment. The electric circuit is created by connecting an anode in the electron-rich anaerobic sediment to a cathode in aerobic water. The figure shows (a) an electric circuit, (b) power output, and (c) applications of the biogeochemical battery. Note that electrons flow from reduced materials at the anode to an electron acceptor (O_2) at the cathode (a) and that the addition of an organic carbon electron source causes a pulse of electric power (b). (Adapted from Bond et al., 2002.)

microorganisms colonizing a cathode are induced to synthesize high-value, multicarbon organic chemicals when electricity is delivered to the bioreactor system (Logan and Rabaey, 2012; Nevin et al., 2010).

Case study 2: hydrogen gas from microbial fermentation

Hydrogen gas has been recognized as a very promising alternative to petroleum-based fuels – especially in automobiles (Logan, 2004; Lee et al., 2010). Hydrogen can be produced from renewable material such as biomass, and hydrogen can be transported and used in internal combustion engines. Furthermore, combustion of H_2 does not contribute to global warming by adding CO_2 to the atmosphere.

As mentioned above and in Section 3.8, anaerobic bacteria (e.g., *Clostridium*) and communities of anaerobic microbial populations are routinely limited by available electron acceptors. Hydrogen gas (H_2) generated by proton-reducing reactions is a common fermentation byproduct generated during electron-acceptor-limited microbial processes.

Ordinarily excess carbonaceous waste products from the food industry are managed in ways that consume, rather than generate, energy. To investigate the feasibility of generating H_2 from food industry waste, Oh and Logan (2005) monitored H_2 production in an anaerobic bioreactor supplied with heat-treated wastewater from a cereal food-processing plant. Hydrogen gas concentration reached a maximum of 32–39% in the bioreactor headspace. If properly engineered and refined, this "biogas" process could become the basis of a practical energy-generation technology. Assuming the organic waste being processed in the fermentation technology is glucose, the maximum theoretical yield of hydrogen gas is 12 moles per mole of glucose:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12\ H_2 + 6CO_2$$

However, very real physiological constraints currently limit the theoretical yield of H_2. Typically 17% of donor electrons flow to proton reduction, yielding 2 mole H_2/mole glucose (Lee et al., 2010). In the future, it is conceivable that genetic engineering of the fermenting microorganisms may boost the hydrogen production yield (Rupprecht et al., 2006).

Case study 3: genetic engineering of photosynthesis-based production of hydrogen gas

Solar energy is unquestionably the most abundant source of energy on Earth. Each year across the void of space, the sun delivers to our planet ~5.7×10^{24} J of radiation (Miyake et al., 1999). This is ~10,000 times more than the total energy consumed by humans. From an evolutionary standpoint, the development of oxygenic photosynthesis (see Sections 2.8 to 2.11) was one of the most innovative of biological events. In searching for

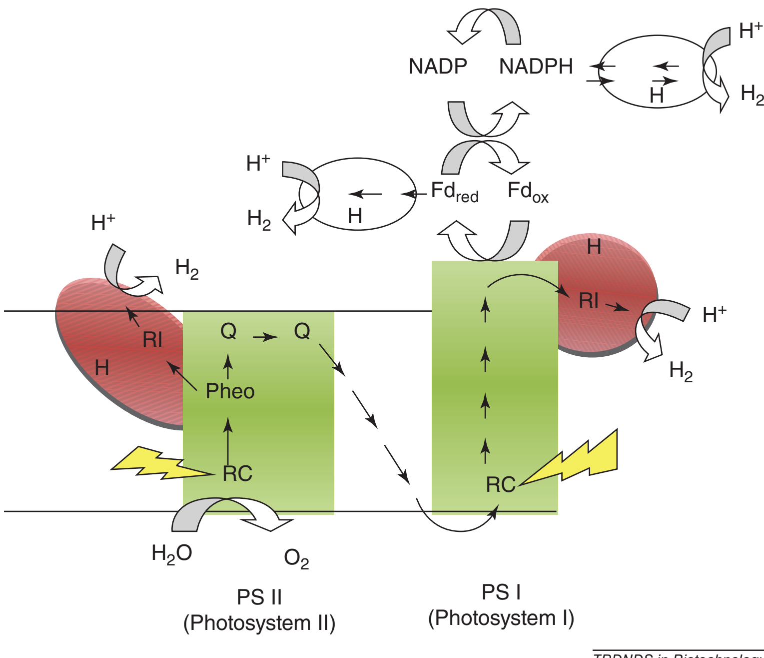


Figure 8.18 Hydrogen production avenues connected to oxygenic photosynthesis and fueled by sunlight (yellow arrows) that is absorbed by the photosystems and transferred to the reaction center (RC). Arrows indicate electron transfer pathways. The hydrogenase complex (H) is indicated in black and white if efficient BioH_2 production has been demonstrated already through this pathway. The complex is indicated in red if the pathway is a potential future avenue of BioH_2 production, such as electron transfer to hydrogenase from pheophytin (Pheo) in PS II or from the electron acceptor side of the PS I complex. Abbreviations: Fd, ferrodoxin; Q, quinone; RI, redox intermediates. (Reprinted from *Trends Biotechnol*. **28**:262–271. Lee, H.-S., W.F.J. Vermaas, and B.E. Rittmann. 2010. Biological hydrogen production: prospects and challenges with permission from Elsevier.)

sources of clean, safe, renewable energy, genetic engineers have ideas of new light-harvesting innovations.

As presented in Chapters 2 and 3 (Sections 2.10, 3.7, and 3.9), the oxygenic photosynthesis key advance was to use energy in light to split water into oxygen and protons – liberating high-energy electrons that can flow through electron transport chains to create the cell's energy currency, ATP. A goal pursued by some genetic and biochemical engineers is to modify electron flow in photosynthesis so that it is directed towards enzymes that synthesize hydrogen gas. Hydrogen gas production (in small quantities) was discovered in oxygenic (photosynthetic) cyanobacteria in the 1890s and in eukaryotic green algae in the 1940s (Prince and Kheshgi, 2005). Though these physiological reactions are a reality, selective evolutionary forces have not led to process optimization. Figure 8.18 illustrates one basic

bioengineering concept that fuses two well-known systems: photosystems I and II (that generate high-energy electrons) and hydrogenases, which use electrons to reduce protons, synthesizing H_2 gas. The hydrogenase enzyme complexes (shown as ovals in Figure 8.18) fall into two categories: (i) proven pathways (in black and white, using reduced ferredoxin or NADPH as electron donors) and (ii) as potential new pathways (in red, where electron flow is diverted directly from photosystems I and II to hydrogenases).

Another well-studied enzyme system that produces hydrogen gas is nitrogenase, whose function demands high levels of ATP (see Section 7.4, Box 7.6). A strategy that is an alternative to that shown in Figure 8.18 would use the anoxygenic photosynthetic bacterium *Rhodopseudomonas palustris* to generate ATP, which, in turn, can drive nitrogenase-based H_2 production using organic substrates as electron donors for proton reduction (Lee et al., 2010). Prince and Kheshgi (2005) have stated that hydrogen production via hydrogenase, instead of nitrogenase, offers advantages that include higher efficiency and elimination of ATP consumption. Although the basic concept of coupling photosynthetic apparatus to proton-reducing apparatus is highly meritorious, there are many details that need to be addressed before the idea becomes a technology. Such details include:

- Which photosystem in which host? (Oxygenic photosynthesis in algae/cyanobacteria or anoxygenic photosynthesis in a bacterium?)
- Which proton-reducing system? (Nitrogenase or hydrogenase?)
- How can the oxygen-sensitive proton-reducing systems (hydrogenase and nitrogenase) be protected from O_2?
- How can photosystem efficiency be boosted?
- How can hydrogen-consuming pathways in host cells be eliminated?
- Will hybrid protein systems assemble and mature and retain their functionality?

Case study 4: genetically engineering the Bt insecticide in crop plants

Bacillus thuringiensis (Bt) is the ubiquitous spore-forming bacterium that produces insecticidal protein crystals called “Bt toxin”. Though the bacterium was discovered in 1901, spores and protein crystals from this bacterium were not used commercially as an insecticide until the 1950s (Glick and Pasternak, 2003). Within the bacterial spore is a “parasporal body” that houses what is known as the “pro-toxin”, an inactive form of the toxin. When the parasporal crystal is ingested by the target insect, proteolytic cleavage releases the active toxin. The toxin inserts itself into the membrane of the gut epithelial cells of the insect, causing excessive loss of ATP through an ion channel. Cellular metabolism ceases, the insect stops feeding, becomes dehydrated, and eventually dies (Glick and Pasternak, 2003).

The genes for Bt toxin have been cloned and transferred to the chloroplasts of many crop plants (e.g., cotton, potatoes). Vast plantings of these crops no longer need to be sprayed with chemical insecticides that may be inefficiently applied and have unanticipated environmental impacts. Though the potential negative environmental impacts of genetically engineered organisms are highly controversial, it is undeniable that Bt crops have had dramatic environmental and economic benefits.

Case study 5: engineering crop plants for herbicide resistance

The herbicide glyphosate is a synthetic compound similar to the amino acid glycine (Figure 8.19). Glyphosate inhibits a key enzyme involved in the biosynthesis of aromatic amino acids in both plants and bacteria. A gene encoding a glyphosate-resistant version of the biosynthetic enzyme was discovered in *E. coli*. That gene was cloned and introduced into plant cells in tobacco, petunia, tomato, potato, soy, maize (corn), sorghum, canola, alfalfa, and cotton crops (among others). The transgenic crops produce a sufficient amount of the enzyme to allow amino acid synthesis to proceed when their own enzyme is inactivated by glyphosate application. The result is a "package deal" from the manufacturer: when glyphosate is applied to farm land, only planted transgenic crops resist the applied herbicide. Again, a number of ethical, economic, social, ecological, and other issues accompany implementation of genetically modified crop technology (often referred to as genetically modified organisms, "GMOs").

CH_2COOH | NH | $H_2C-P(=O)(OH)-OH$

glyphosate (herbicide)

CH_2COOH | NH_2

glycine (amino acid)

Figure 8.19 Structures of the herbicide, glyphosate, and the amino acid, glycine.

Case study 6: genetic engineering of plants to detoxify mercury

In Chapter 7 (Sections 7.1 and 7.3), we discussed interactions between microorganisms and mercury. Some of the interactions were also mentioned as mercury bioremediation strategies in Section 8.3. An elaboration of the mercury biogeochemical cycle is depicted in Figure 8.20. Mercury is found in the naturally occurring mineral, cinnabar, and in other minerals. Mercury has many industrial uses – in, for example, thermometers, biocides, industrial catalysts, and gold-mining operations. Unfortunately, mercury is also a highly toxic neurotoxin and impairs kidney function. Methyl mercury impedes biochemical reactions by binding to DNA, RNA, and sulfhydryl groups in proteins. While the cationic form of mercury Hg(II) is likely the most environmentally abundant, it is converted to the highly toxic methyl mercury (CH_3Hg^+), largely by sulfate-reducing populations in aquatic sediments. As mentioned in Sections 7.1 and 7.3, pure cultures of bacteria capable of detoxifying (mobilizing) Hg(II) and CH_3Hg^+ have been

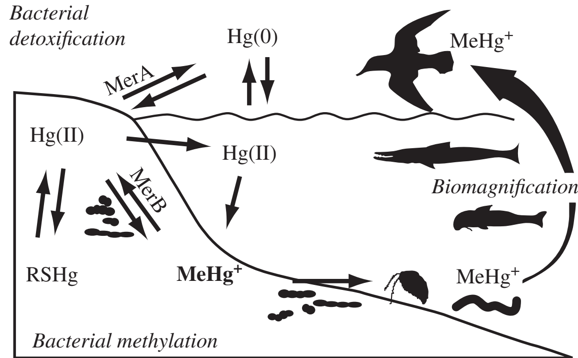


Figure 8.20 The mercury biogeochemical cycle. $MeHg^+$, methyl mercury; RSHg, mercury bound to organic molecules via sulfur. (Reprinted by permission from Macmillan Publishers Ltd: *Nature Biotechnology*, from Bizily, S.P., C.L. Rugh, and R.B. Meagher. 2000. Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnol.* **18**:213–217. Copyright 2000.)

characterized, and genes encoding the key reactions (Figure 8.21) have been cloned and sequenced; *merB* encodes the enzyme (organomercurial lyase) that converts methyl mercury to Hg(II). Furthermore, *merA* encodes the enzyme (mercuric reductase) that reacts with reduced nicotinamide adenine dinucleotide phosphate (NADPH) to convert Hg(II) to elemental mercury Hg(0).

$$R\text{–}CH_2\text{–}Hg^+ + H^+ \xrightarrow{\text{MerB}} R\text{–}CH_3 + Hg(II)$$

$$Hg(II) + NADPH \xrightarrow{\text{MerA}} Hg(0) + NADP^+ + H^+$$

Figure 8.21 The bacterial mercury-processing enzymes, organomercurial lyase (MerB) and mercuric reductase (MerA), catalyze the reactions shown.

In order to investigate the feasibility of using plants to detoxify mercury-contaminated soils and sediments, Bizily et al. (2000) transferred and expressed *merAB* in *Arabidopsis thaliana* (a small flowering plant related to cabbage that is easily amenable to genetic engineering). When grown hydroponically, not only did the transgenic plants resist toxic concentrations of CH_3Hg^+ and Hg(II) but they also converted these compounds to Hg(0). These data establish the principle that transgenic plants have the potential to grow in mercury-contaminated soils and mobilize mercury out of that habitat. Presumably, once removed from soil, the vapor-phase Hg(0) would be scrubbed and recovered from the air (Ruiz and Daniell, 2009).

Case study 7: biodegradable plastic

Carbonaceous, cellular reserve storage products that guard against starvation in bacteria (see Section 3.5) were described as early as 1926 (Lenz and Marchessault, 2005; Keshavarz, and Roy, 2010**)**. Polyhydroxyalkanoates (PHAs; Figure 8.22) occur as high molecular weight (mass of 50–100 kDa)

storage bodies in many types of Gram-positive and Gram-negative bacteria (Figure 8.23). At least 75 different genera of bacteria have been shown to produce PHAs and these exhibit many structural variations. The R group shown in Figure 8.22 symbolizes an akyl side chain that varies in length and branching mode so that more than 100 different monomer units have been identified as constituents of PHA (Reddy et al., 2003).

PHAs are nontoxic polymers that are biocompatible (nonallergenic, nontoxic) and biodegradable. They can be produced from renewable resources and feature many traits similar to modern synthetic plastics: a high degree of polymerization, highly crystalline, optically active, and insoluble in water. One remarkable bacterium, *Ralstonia eutrophus*, is capable of channeling as much as 90% of its dry weight into PHA. Moreover, the chemical composition (hence physical traits) of PHA polymers can be manipulated by varying the organic substrates fed to the PHA-producing bacteria. For instance, the PHA co-polymer formed by *R. eutrophus* grown on a mixture of

$$\left[-O-\overset{\displaystyle R \atop |}{CH}-CH_2-\overset{\displaystyle O \atop \|}{C}- \right]_n$$

Figure 8.22 Molecular structure of polyhydroxyalkanoate molecules that constitute biodegradable plastics. (Reprinted from Reddy, C.S.K., R. Ghai Rashmi, and V.C. Kalia. 2003. Polyhydroxyalkanoates: an overview. *Bioresource Technol*. **87**:89–101. Copyright 2003, with permission from Elsevier.)

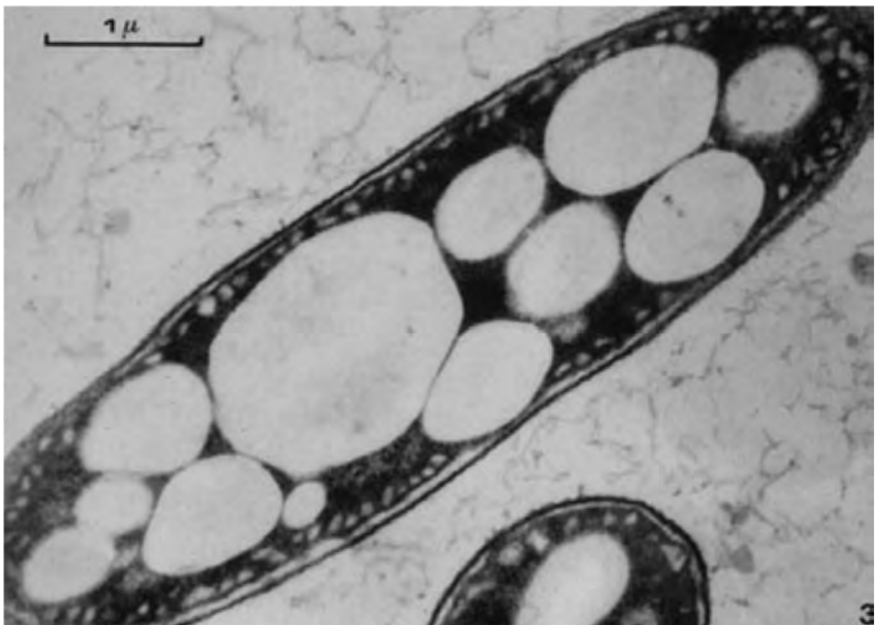


Figure 8.23 Photomicrograph of polyhyroxyalkanoate bodies within bacterial cells. This transmission electron micrograph of an ultrathin section shows *Azotobacter chroococcum*. (Reprinted with permission from Lenz, R.W. and R.H. Marchessault. 2005. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *BioMacromolecules* **6**:1–8. Copyright 2005, American Chemical Society.)

$$\left[-O-\underset{CH_3}{\overset{H}{C}}-CH_2-\overset{O}{\overset{\|}{C}}- \right]_m \left[-O-\underset{C_2H_5}{\overset{H}{C}}-CH_2-\overset{O}{\overset{\|}{C}}- \right]_n$$

HB HV

Figure 8.24 Structure of the microbially synthesized plastic co-polymer, hydroxybutyrate (HB) hydroxyvalerate (HV). (Reprinted with permission from Lenz, R.W. and R.H. Marchessault. 2005. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *BioMacromolecules* **6**:1–8. Copyright 2005, American Chemical Society.)

glucose and propionic acid is a random polyester of hydroxybutyrate (HB) and hydroxyvalerate (HV; Figure 8.24). The HB–HV co-polymer has a lower melting point than an HB homopolymer – conferring better mechanical and processing characteristics.

As early as 1982, Imperial Chemical Industries (ICI) in the UK announced a product development program on PHA-based biodegradable plastics – creating the plastic containers dubbed "Biopol" (Figure 8.25). Over the past two decades, other corporations in the USA and Japan have created biodegradable plastic products ranging from packaging materials to agricultural mulch film to resins. Overall, three types of biodegradable plastics have been introduced: photodegradable, semibiodegradable (a hybrid of starch-based polymer and polyethylene), and completely biodegradable (Reddy et al., 2003).

One production strategy for PHA-based plastics is to use industrial bioreactors (fermentors) to grow PHA-synthesizing heterotrophic bacteria on carbon sources ranging from glucose to methanol to cane molasses to cheese whey. Clearly the economic viability of PHA-based plastic

Figure 8.25 Commercially manufactured, biodegradable, molded plastic containers created from microbial polyesters. (Reprinted with permission from Lenz, R.W. and R.H. Marchessault. 2005. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *BioMacromolecules* **6**:1–8. Copyright 2005, American Chemical Society.)

manufacturing is dependent upon many factors – including the costs of the bacterial growth substrates. Another strategy for PHA-based plastics involves transgenic plants. The biochemical pathways for PHA biosynthesis begin with the intermediary metabolite, acetyl coenzyme A (CoA). Three enzymes encoded by the gene cluster *phbCAB* create the PHA polymer. The *phbCAB* gene cluster has been cloned and expressed in plastids of *Arabidopsis thaliania* – successfully producing the PHA polymer (Reddy et al., 2003). For potential commercialization in plants, oil seed crops (e.g., rape seed, sunflower) and starch crops (e.g., corn) represent good prospects for large-scale agricultural production of PHA plastics.

According to Keshavarz, and Roy (2010), PHAs have rapidly gained interest both in research and industry. Their structural versatility and characteristics have been investigated and new areas of exploitation are being discovered. Patents have been issued related to a range of applications of PHAs in molding, containers, pens, golf tees, diapers, personal hygiene materials, hot-melt and pressure-sensitive adhesives, films, flavor delivery agents in foods, dairy cream substitutes, and fabrics and materials for manufacturing compostable articles and solvents. More recently attention has focused on the medical applications of PHAs. The major drawback for extensive use of these polymers is their high production cost. In this regard, research is continuing on their production from inexpensive raw materials including varieties of waste and byproducts. For waste products to be used extensively, the consistency and reliability of the raw material, storage issues, and the correct balance of the ingredients will need to be scrutinized carefully. The potential of different bacterial species and recombinant strains will be explored further in the context of increased PHA yield and productivity and their application as blends of plastics and composite materials (Keshavarz, and Roy, 2010). In the end, marketplace issues, such as consumer demand and the price of traditional plastics, will govern the destiny of biodegradable plastic products.

Case study 8: environmental epidemiology of a newly emerged disease, SARS

Severe acute respiratory syndrome (SARS) erupted late in 2002, eventually sickening thousands of people around the globe. The illness was first reported in the Guangdong Province of the People's Republic of China where several hundred people exhibited a severe and unusual form of pneumonia. After similar cases were detected in patients in Hong Kong, Viet Nam, and Canada during February and March 2003, the World Health Organization (WHO) issued a global alert for the illness designated "SARS" (Rota et al., 2003; Skowronski et al., 2005). Transmissibility of SARS became obvious when healthcare workers and household members who had cared for SARS patients in Hong Kong and Viet Nam also exhibited symptoms. Many of the SARS cases could be traced to contacts with a single healthcare worker from Guangdong Province who visited Hong

Kong, where he was hospitalized with pneumonia and died. The disease spread at an alarming rate and had an equally alarming fatality rate: by the end of April 2003, >4300 SARS cases and 250 SARS-related deaths had been reported to the WHO from over 24 countries. In some instances, death due to respiratory failure reached 10%. All told, 800 people died.

- **What was the origin of the new disease?**

Answer: It had "jumped" from animals to humans.

- **What tools are available to characterize the disease and its causative agent?**

Answer: Epidemiology, culture-based isolation of the infectious agent, genomics, and structural biology.

In an extraordinary effort, the WHO organized a network of 13 laboratories in 10 countries. These teams identified a virus (by repeated cultivation in human tissue-culture cells) associated with SARS during the third week of March 2003. Two weeks later, the entire genome of the causative agent, a caronavirus, was sequenced (~30,000 bp; Marra et al., 2003).

It was no surprise that the type of virus associated with SARS was a caronavirus. Carona viruses are RNA viruses with a long history of causing respiratory and intestinal diseases in humans and other animals. Typically, they have narrow host ranges. Genomic analysis of the virus indicated that the virus arose by ancient recombination(s) with subsequent evolution, including deletional mutation events, over time (Skowronski,et al., 2005).

- **So where did the new virus come from?**
- **What was present in the Guangdong Province of China that allowed the SARS pathogen to "emerge"?**

The current thought is that masked palm civet cats (Figure 8.26), traded in large, open-air markets of China, were the source of the caronavirus. More than 13% of approximately 500 animal traders from Guangdong Province tested positive for the virus in blood samples taken during the initial SARS outbreak. (In control groups only 1–3% of animal traders tested positive.) Domesticated cats and ferrets are other animals in Guangdong Province that are easily infected by the virus and routinely contact humans. Some public health experts have recommended that China develop new strict regulations on commercial animal handling and trading that may curb emergence and spread of disease in the future. Although the civet cats served as vectors that led to human infection and the SARS outbreak, the long-term natural reservoirs for SARS-like viruses are bats (horseshoe bats) that feed on tree fruits in rural China.

- **But what allowed the host range of the SARS virus to broaden from civet cats to humans?**

Figure 8.26 Photograph of the masked palm civet cat traded in open-air markets in China. These animals are thought to be a likely source of the SARS virus. (Courtesy of Robert Siegel, Stanford University, with permission.)

Genomic sequence comparisons showed that viruses specific to animals and humans differed by only a handful of mutations. Li et al. (2005) analyzed the influence of a change in one protein in the SARS virus that seems critical for recognition of its human host. The crystal structure of a spike-shaped viral recognition protein was analyzed while in physical contact with its attachment (and fusion) site on human cells. By examining the details of molecular bonding in the virus–human complex, Li et al. (2005) discovered that two amino acids differ from those in strains of the animal viruses unable to infect humans. It is thought that the conformational change effected by the new amino acids allowed the altered animal virus to recognize its (new) human host. New information about the structural mechanism for virus–host recognition may assist in the development of caronavirus vaccines that prevent infection (Li et al., 2005).

Thus, information from a multitude of sources (natural history, epidemiology, structural biology, and genomics) converged to help explain the emergence of SARS.

Case study 9: 2010 cholera outbreak in Haiti

Cholera appeared in Haiti in October 2010 for the first time in recorded history (Frerichs et al., 2012). The causative agent was quickly identified by the Haitian National Public Health Laboratory and the United States Centers for Disease Control and Prevention as *Vibrio cholerae* serogroup O1, serotype Ogawa, biotype El Tor. *Vibrio cholerae* colonizes the small intestine, attaching to the epithelial wall where the bacterium grows and releases a toxin. The toxin causes loss of fluids (~20 liters per day!). Mortality can be as high as 60%. Treatment involves replacement of fluids and administering antibiotics. During the course of the Haitian epidemic, >500,000 government-acknowledged cholera cases and >7000 deaths occurred: the largest cholera epidemic in the world (Frerichs et al., 2012). Questions of origin were widely debated with some attributing the onset of the epidemic to climatic factors and others to human transmission. None of the evidence on origin supported climatic factors.

Pooling information based on epidemiological and molecular-genetic evidence led to the conclusion that the source of the outbreak was United Nations peacekeeping troops from Nepal, having reached Haiti in October, 2010. Crucial to this diagnosis was next-generation whole-genome sequencing (Pacific Biosciences; see Section 6.10). This technology was used to determine the genome sequences of 2 clinical *Vibrio cholerae* isolates from the outbreak in Haiti, 1 strain that caused cholera in Latin America in 1991 and 2 strains isolated in South Asia in 2002 and 2008 (Chin et al., 2011). Primary DNA sequence data allowed the comparison of the genomes of these 5 strains, along with a set of previously obtained partial genomic sequences of 23 diverse strains of *V. cholera*. Fine-scale genetic patterns (polymorphisms) identified the Asian source of the cholera outbreak (Chin et al., 2011). Such findings have important policy implications for shaping future international relief efforts. Safeguards should be taken to prevent the release of human pathogens into highly susceptible communities.

8.7 ANTIBIOTIC RESISTANCE

Our shield against infections

A revolutionary advancement in modern medicine was the discovery of antibiotics – compounds that can be taken orally or intravenously by humans to destroy microbial agents of disease. Using the lexicon from werewolf folklore, antibiotics are "silver bullets" – able to act selectively on target organisms, while sparing the infected host. The antibiotic revolution was initiated by A. Fleming's 1929 discovery of penicillin (which interferes with cell wall synthesis primarily in Gram-positive bacteria)

and by the 1944 discovery by A. Schatz and S. Waksman of streptomycin (which interferes with ribosomal protein synthesis in both Gram-negative and Gram-positive bacteria). Prior to antibiotics, chemotherapy for microbial infections relied on sulfa drugs (e.g., sulfonamides, often allergenic in humans) and arsenic-containing compounds (often toxic to humans).

An appreciation of the impact of antibiotics on contemporary human wellbeing can be developed by imagining a world without antibiotics.

- **How safe would you and your doctor feel if pharmacies no longer filled prescriptions for antibiotics?**
- **What if the antibiotics we routinely take to combat disease lost their effectiveness?**

Table 8.10 provides a listing of the many diseases and bacterial infectious agents that have the potential to threaten human health in the United States and other countries. Figure 8.27 illustrates the impact of antibiotics on the US population by displaying the annual rate of mortality caused by infectious disease (per 100,000 people per year) from 1900 to 1992. Prominent features of the graph are:

- The death rate from infectious disease has plummeted dramatically.
- A short-lived spike in the death rate occurred in 1918 that is attributable to the 1918 influenza pandemic.

The decline in infectious disease-caused death reflects several factors, such as improved medical science, hygiene, sanitation, personal care, and standard of living (Lederberg, 1997; Armstrong et al., 1999). However, a major contribution to the decline was the clinical use of sulfa drugs (1935), penicillin (1941), and streptomycin (1943). In the early years, demand for antibiotics in the treatment of infectious disease often far exceeded supply. For example, during World War II, penicillin was in such scarce supply that it was routinely recovered from the urine of patients in hospital wards and then reused.

A shield that needs constant renewal

Antibiotics are effective in thwarting disease-causing agents because the biochemical structure and function of the agents are distinctive from those of humans. In Chapter 2 (Section 2.12, Table 2.3) we reviewed some of the major biochemical distinctions between *Eukarya*, *Archaea*, and *Bacteria*. What arose during evolution translates into drug design strategies that are crucial for medicine and public health. Key cellular targets for antibiotics in *Bacteria* are cell wall synthesis, nucleic acid replication, protein synthesis,

Table 8.10
Reported bacterial diseases and infectious agents in the United States. (Modified from Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 833. Prentice Hall, Upper Saddle River, NJ. Copyright 2006. Reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ)

Anthrax	Pertussis
Botulism	Plague
Brucellosis	Psittacosis
Chancroid	Q fever
Chlamydia trachomatis	Rocky Mountain spotted fever
Cholera	Salmonellosis
Diphtheria	Shigellosis
Ehrlichioss	Streptococcal diseases, invasive, group A
Enterohemorrhagic *Escherichia coli*	Streptococcal toxic shock syndrome
Escherichia coli O147:H7	*Streptococcus pneumoniae*, drug-resistant and invasive disease
Gonorrhea	Syphilis
Haemophilus influenzae, invasive disease	Tetanus
Hansen's disease (leprosy)	Toxic shock syndrome
Hemolytic uremic syndrome	Tuberculosis
Legionellosis	Tularemia
Listeriosis	Typhoid fever
Lyme disease	Vancomycin intermediate *Staphylococcus aureus* (VISA)
Meningococcal disease	Vancomycin-resistant *Staphylococcus aureus* (VRSA)

Viral-, fungal-, protozoan-, and helminth-caused infections were omitted from this list. These agents of disease pose special challenges for development of selective therapeutic agents because they rely on eukaryotic-type cellular metabolism.

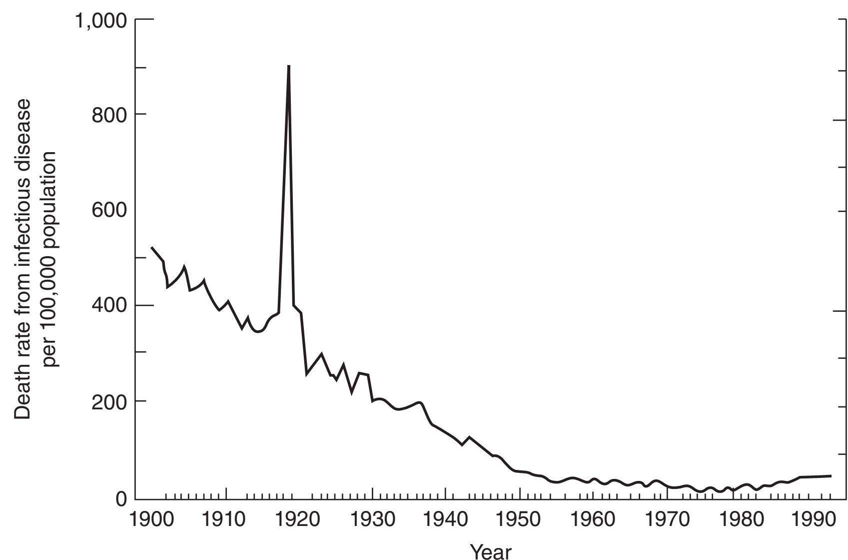


Figure 8.27 Trends in infectious diseases mortality (annual death rate per 100,000 people) 1900–1992. (Source: Lederberg (1997) and Centers for Disease Control.)

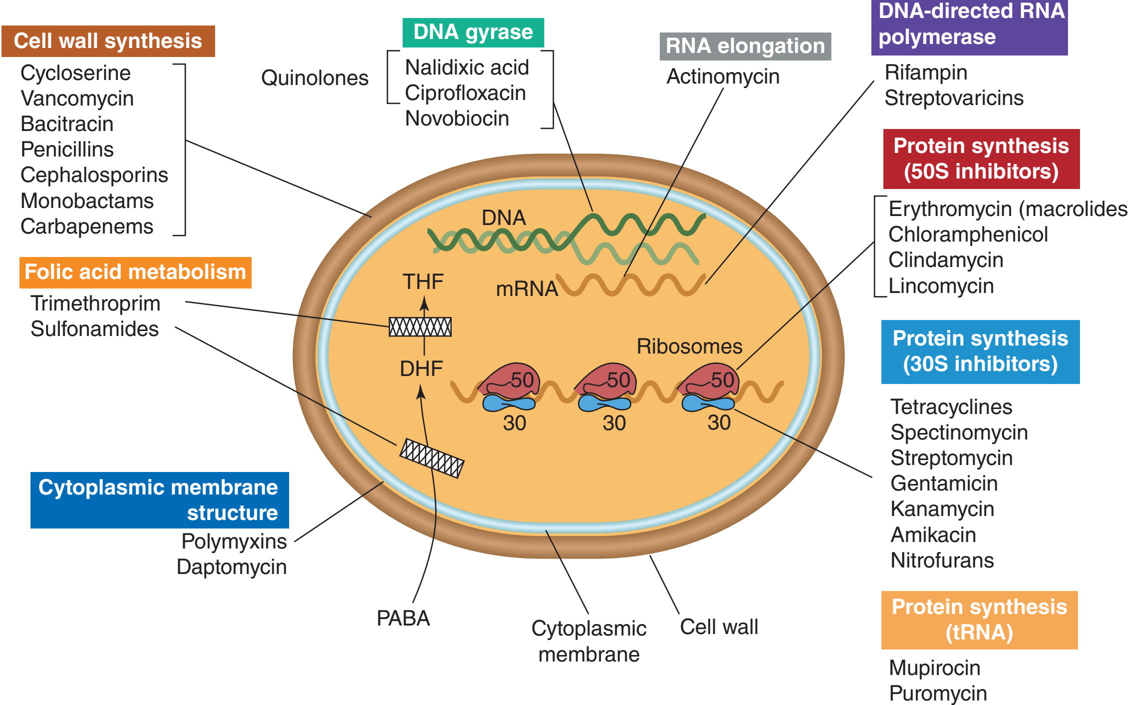


Figure 8.28 Mode of action of major antimicrobial agents. DHF, dihydrofolate; DNA, deoxyribonucleic acid; mRNA, messenger RNA; PABA, para-amino benzoic acid; THF, tetrahydrofolate; tRNA, transfer RNA. (From Madigan, M. and J. Martinko. 2006. *Brock Biology of Microorganisms*, 11th edn, p. 683. Prentice Hall, Upper Saddle River, NJ. Copyright 2006. Reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ.)

cytoplasmic membrane, and folic acid metabolism (the latter is essential for precursors for nucleic acid biosynthesis). Information in Figure 8.28 provides a summary of the modes of action of 30 antibiotics in widespread use today (Madigan et al., 2014).

However, there is a problem: antibiotics that were effective yesterday, become ineffective today. Table 8.11 displays the deployment dates of 10 antibiotics and the dates that microorganisms were found to be resistant to each (Palumbi, 2001). Antibiotic-resistant disease-causing microorganisms are a serious concern for us all. As one antibiotic loses effectiveness, it must be replaced by another. Thus, our shield against diseases is a slowly eroding one that needs constant renewal.

Mechanisms of antibiotic resistance

Each antibiotic targets a specific cellular function (such as inhibiting peptidoglycan biosynthesis or altering membrane sterols to increase cell permeability). In the face of selective pressure for survival, bacteria have responded with adaptations that resist antibiotics in eight different ways

Table 8.11
Dates of deployment of representative antibiotics and the evolution of resistance. (From Palumbi, S.R. 2001. Humans as the world's greatest evolutionary force. *Science* **293**:1786–1790. Reprinted with permission from AAAS)

	Evolution of resistance to antibiotics	
Antibiotic	**Year deployed**	**Year resistance observed**
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	Late 1960s

(Table 8.12; Davies, 1994). Key mechanisms of antibiotic resistance include: efflux pumps that reduce the intracellular concentration of the antibiotic, enzymes that inactivate the antibiotic, and alteration of the structure of the cellular site targeted by the antibiotic (Table 8.12).

The eight mechanisms of antibiotic resistance are phenotypes. The way to thwart the development of such phenotypes is to understand their underlying genetic basis. The critical questions that address the vicious cycle between new antibiotic deployment and microbial resistance are:

1. How do new *genotypes* for drug resistance arise?
2. How do the genotypes become widely distributed among pathogenic microorganisms?

The antibiotic resistance gene pool

As stated clearly by Davies (1994), Davies and Davies (2010), Martinez (2008), and documented convincingly by D'Costa et al. (2006), antibiotic-producing microorganisms that are native to natural habits (such as soil) constitute a major reservoir for genes that encode antibiotic resistance. For decades, the filamentous Gram-positive streptomycetes have been used by pharmaceutical companies as a source of antibiotic discovery and production. These same organisms have self-protection mechanisms. D'Costa et al. (2006) isolated 480 *Streptomyces* bacteria and screened them for resistance to 21 different antibiotics or drugs (including all major types and targets of activity, some having seen clinical use for decades and some brand new). The results were chilling:

- Every *Streptomyces* strain was resistant to multiple drugs (the average number of resistances was seven or eight).

Table 8.12

Mechanisms of antibiotic resistance in bacteria. The genes for each of these resistance traits can be transferred between bacteria. (From Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**:375–382. Reprinted with permission from AAAS)

Mechanism	Antibiotic
–Reduced uptake into cell	Chloramphenicol
–Active efflux from cell	Tetracycline
–Modification of target to eliminate or reduce binding of antibiotic	β-Lactams (e.g., penicillin G, amoxicillin) Erythromycin, lincomycin
–Inactivation of antibiotic by enzymic modification:	
Hydrolysis	β-Lactams
	Erythromycin
Derivatization	Aminoglycosides
	Chloramphenicol
	Fosfomycin
	Lincomycin
–Sequestration of antibiotic by protein binding	β-Lactams
–Metabolic bypass of inhibited reaction	Fusidic acid
–Binding of specific immunity protein to antibiotic	Sulfonamides
	Trimethoprim
	Bleomycin
–Overproduction of antibiotic target (titration)	Sulfonamides
	Trimethoprim

- Two strains were resistant to 15 of the 21 drugs.
- Resistances were found for both naturally occurring and for chemically synthesized antibiotics.
- Many of the resistances were linked to enzymatic inactivation of the antibiotics.

While self-protection mechanisms in streptomycetes have long been known (the producer *needs* to be resistant), the breadth of genes conferring multiple drug resistance and their effectiveness on antibiotics not yet in wide clinical use were sobering.

- **Do mutation and recombination also contribute to the genetic pool of antibiotic resistance?**

Answer: Yes. For example, J. Davies (1994) has stated that the role of mutation is especially important in the evolution of resistance to β-lactam antibiotics, such as penicillins and cephalosporins. The precise mechanisms by which antibiotic-resistance genes and gene clusters evolve have yet to be discovered. However, the scenario described in Section 8.5 for the evolution of new metabolic pathways (gene duplication, mutation, recruitment, recombination, and selection) generally applies.

In summary, there are eight mechanisms of antibiotic resistance (Table 8.12) and their underlying genetic bases are derived from both short-term mutation/selection and a large long-standing reservoir of antibiotic-producing microorganisms in nature. The question "How do the genotypes become widely distributed among pathogenic microorganisms?" is addressed next.

Dissemination of resistant genes

It is one thing to be aware that genetic mechanisms of antibiotic resistance may exist in some soil-dwelling strain of *Streptomyces* in some remote location. It is quite another thing to know that the host of a mobile genetic element (e.g., plasmid or transposon) conferring resistance to vancomycin is here in the hospital in patients being treated for an infectious disease with vancomycin. The first scenario is cause for mild concern. The latter scenario is alarming.

Even though there are many barriers against facile exchange of genetic material between taxonomically unrelated microorganisms (e.g., low frequency of contact, surface exclusion, enzymes that digest foreign DNA; Thomas and Nielsen, 2005), retrospective studies prove that it does occur (see Section 5.9). Like mutations, horizontal gene transfer frequencies may be rare, but if the selective pressure for such rare events is strong, then the new genetic combinations (host plus transferred gene(s)) will flourish. *Thus, it is crucial to realize that proliferation of antibiotic resistance requires two on-going events*:

1. The transfer itself.
2. Selective pressures that allow the transferred genes to proliferate in the gene pool.

Davies (1994) has provided a model for the acquisition of genetic determinants for antibiotic resistance in pathogenic bacteria (Figure 8.29). The model begins (top of Figure 8.29) with an antibiotic-resistance gene pool (whose origins were addressed above). The four fundamental steps in the dissemination of resistance genes across taxonomic boundaries to pathogens are:

1. Uptake into cell cytoplasm of the resistance-conferring genetic elements; the best characterized hypothetical mechanisms are transformation (uptake of naked DNA), conjugation (intercellular delivery of plasmids between adjacent cells), and transduction (virus-mediated genetic exchange).
2. Formation of small multidrug-resistant mobile genetic elements. The examples of mobile genetic elements shown in Figure 8.29 are integrons. Integrons have been discovered to be widespread in nature. They are mobile genetic elements with a relatively conserved structure that includes an enzyme (integrase) that mediates insertion into host DNA, a "cassette" area that can carry and receive antibiotic-resistance genes, and several promoters that activate gene expression.

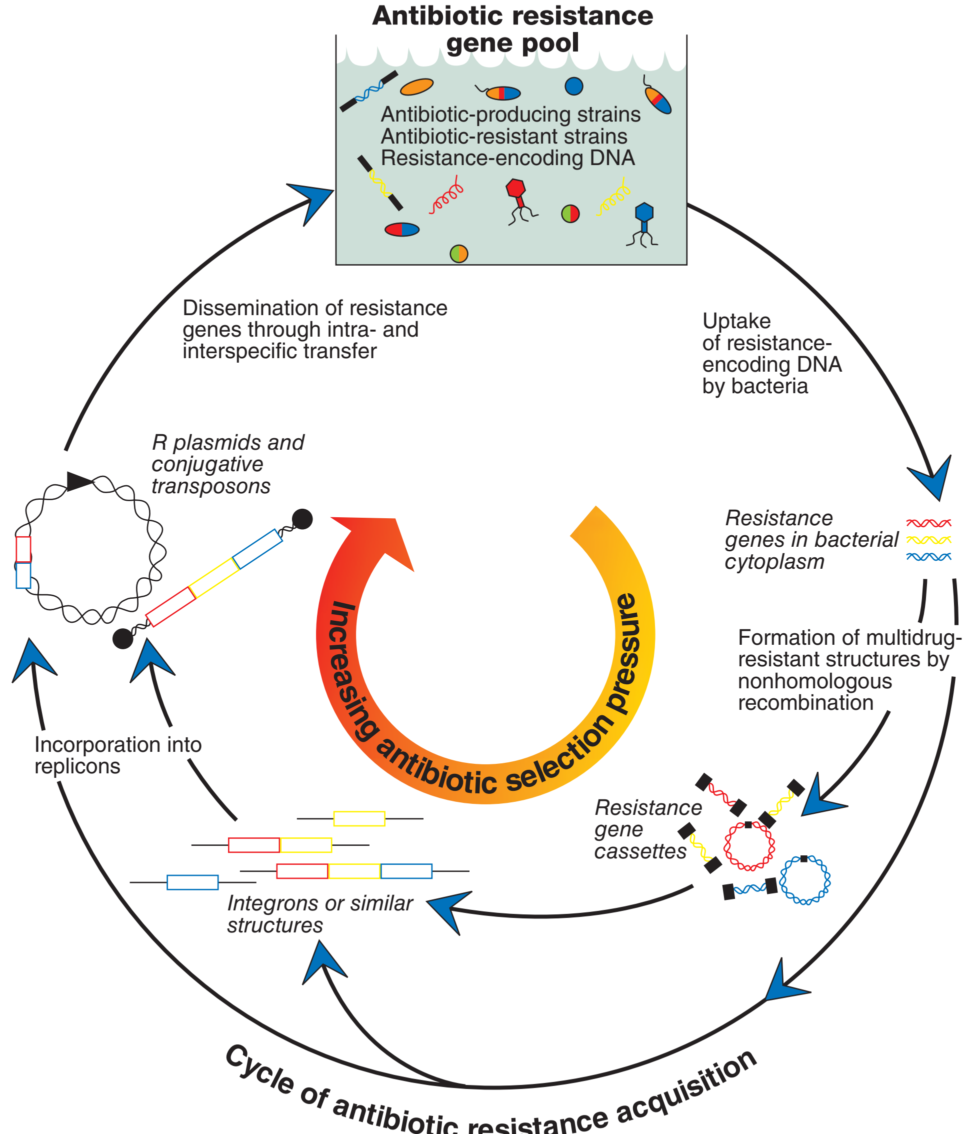


Figure 8.29 A scheme showing the route by which antibiotic resistance genes are acquired by bacteria in response to the selection pressure of antibiotic use. The resistance gene pool represents all potential sources of DNA encoding antibiotic resistance determinants in the environment. This includes hospitals, farms, or other places where antibiotics are used to control bacterial development. After uptake of single- or double-stranded DNA by the bacterial host, the incorporation of the resistance genes into stable replicons (DNA elements capable of autonomous replication) may take place by several pathways that have not yet been identified. The involvement of integrons, as shown here, has been demonstrated for a large class of transposable elements in a group of bacteria known as the Enterobacteriaceae. The resulting resistance plasmids could exist in linear or circular forms in bacterial hosts. The final step in the cycle – dissemination – is brought about by one or more of the gene transfer mechanisms that commonly include transduction, transformation, and/or conjugation (see text for details). (From Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**:375–382. Reprinted with permission from AAAS.)

3 Intracellular incorporation of the small multidrug-resistance cassettes into larger replicating elements such as plasmids.
4 Dissemination of the resistance-encoding DNA back to microbial communities that constitute the antibiotic-resistant gene pool.

Very recently, the notion of an "antibiotic resistome" has been developed (Gillings, 2013; Sommer et al., 2009; Sommer and Dantas, 2011; Forsberg et al., 2012). The resistome is defined as the complement of all antibiotic-resistance genes encoded by a microbial community. To further characterize the genetic foundation of the antibiotic resistome in soil bacteria, Forsberg et al. (2012) applied an unusual mixture of cultivation-based and metagenomic approaches. These authors isolated 95 soil-derived cultures with high-level resistance to a broad array of antibiotics. After pooling DNA extracts from this group of cultures, Forsberg et al. (2012) prepared a large library of cloned DNA fragments (in *E. coli*; see Section 6.9) that were screened on media containing 18 different antibiotics. Sequencing of the cloned, antibiotic-conferring fragments led to the identification of 110 resistance genes. Sixteen of these sequences (encoding resistance to five classes of antibiotics – β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) had 100% nucleotide identity to antibiotic-resistance genes previously sequenced from pathogenic bacteria isolated from hospital patients. The high sequence identity discovered among the shared genes included noncoding regions of DNA (that have no selection pressure to be conserved), as well as many gene sequences from transposons and other DNA-mobilization elements (Forsberg et al., (2012). The above set of findings constitutes convincing evidence of recent genetic exchange of antibiotic-resistance between soil-dwelling microorganisms (the soil resistome) and clinical pathogens.

An urgent need to curb development of antibiotic resistance

There is an overwhelming consensus among experts today that the current (and developing) arsenal of antimicrobial therapeutic agents may soon fail to keep pace with the microbial adaptation to antibiotics. Table 8.13 provides excerpts from three authoritative reports describing the improper use of antibiotics. Such calls for "restrained, proper use of antibiotics" are largely from the medical and public health professionals who rely on antimicrobial therapy to cure human disease. The concern is urgent because the ineffectiveness of antibiotic treatments seems to be accelerating. Resistance to antibiotics in microorganisms is caused by two simultaneous factors:

$$\text{(Mobile resistance genes)} \times \text{(Selective pressure from released antibiotics)} = \text{Widespread resistance}$$

In the above equation, humans have little control over spontaneous horizontal gene transfer and evolution in nature. In contrast, the daily actions of people (individual citizens, doctors, healthcare workers, veterinarians, livestock managers, the poultry industry, the swine industry,

Table 8.13

Calls from the scientific and medical professions for reform in the use of antimicrobial compounds so that they remain effective. (Compiled from Bell, 1998; Levy 2005; Weber, 2005)

Source	Message
Journal of the American Medical Association, 2005. Editorial: "Appropriate use of antimicrobial drugs. A better perspective is needed", by J.T. Weber	• Doctors and patients may be inappropriately overusing antibiotics. This may hasten the emergence of antibiotic resistance in pathogenic bacteria. Efforts to curb inappropriate use of antibiotics should be strengthened
Center for Disease Control. *Emerging Infectious Diseases*, 1998. "Controversies in the prevention and control of antimicrobial drug resistance", by D. Bell	• In hospitals: antimicrobial resistance is spread via high rates of antimicrobial drug use and inadequate infection-control processes • In communities: antibiotics may be prescribed when not required – partly to meet patient demands and partly to be "safe, rather than sorry" • In veterinary medicine: both medical and nonmedical use of antibiotics should be reduced • In developing countries: there are problem in drug resistance in treating diarrheal diseases, sexually transmitted diseases, pneumonia, tuberculosis, malaria, and hospital infections; resources for surveillance and control of antibiotic resistance are inadequate • In clinical laboratories: pathogenic bacteria isolated from diseased hospital patients need to be more carefully screened for low-level resistance to antibiotic therapy
Advanced Drug Delivery Reviews, 2005. "Antibiotic resistance – the problem intensifies", by S.B. Levy	• The frequency of antibiotic-resistant bacteria is increasing – especially worrisome is the emergence of new combinations of multidrug resistance in patients suffering from infectious disease

farmers, marketers, pharmacists, the pharmaceutical industry, the agricultural industry) have a direct impact on releasing antibiotics to our bodies, animals, soils, sediments, and waters where such releases foster an antibiotic-resistance gene pool.

Vast amounts of antibiotics are released

If alive today, Fleming, Schatz, and Waksman would be shocked at the tonnage of antibiotics produced and disseminated globally. Antibiotic use has expanded from therapeutic cures to prophylactic disease prevention, to promotion of growth in the large-scale farming of animals (Table 8.14). Thus, commercial markets for antibiotics in human health care, veterinary medicine, and food production industries are immense. One source (Wenzel and Edmond, 2000) states that in the United States each year, 160 million prescriptions are written for humans and 23 million kg (23,000 metric tons) of antibiotics are used – approximately 50% used by human patients and 50% used in animals, agriculture, and aquaculture. Thus, the

Table 8.14
Medical and commercial uses of antibiotics. (Compiled from Levy, 2002)

Type of antibiotic use	Reason	Type of industry
Therapeutic	Remedial treatment of disease	Human medicine, veterinary
Prophylaxis	Disease prevention	Human medicine, veterinary, aquaculture, honey bees, agriculture
Animal growth promotion	Subtherapeutic doses to promote growth in animals	Livestock and food production

US population of 300 million people received an average of 30 prescriptions per 100 persons per year and ~4 kg of antibiotics per 100 persons per year (Wenzel and Edmond, 2000). Another source (Levy, 2002) states that of the 8 billion animals (especially chickens, turkeys, cattle, and pigs) raised for human consumption in the United States, most receive some antibiotics during their short lifetimes. In the 1950s, use of subtherapeutic levels of antibiotics on animals was found to improve animal growth. When considered in terms of nationwide use today, the amount of subtherapeutic antibiotic usage in animals is 4–5 times that used for treatment of animal diseases (Levy, 2002). Thus, there is no question about the enormity of antibiotic use and release.

Can and should antibiotic releases be curtailed?

Unequivocally, the answer to this question is "Yes". Organizations ranging from the World Health Organization (WHO), the Centers for Disease Control (CDC), to the Alliance for Prudent Use of Antibiotics (APUA) have mounted public campaigns to educate all components of society that control the use and release of antibiotics (institutions, industries, businesses, doctors, farmers, healthcare workers, individuals). Control of antibiotic resistance is a *social problem* of global proportion where major economic, cultural, scientific, and governmental forces meet. The two basic principles of wise antimicrobial usage are as follows:

1. Always administer antibiotics at a dosage and for a period of time that eliminates the pathogens.
2. If the compounds are administrated at inadequate potency, for too short a time, or for the wrong disease, there will be an increased likelihood that pathogens will not be killed. Instead, they may adapt and replicate, and add new traits to the growing pool of antibiotic-resistance mechanisms that erode our shield against infectious disease.

The issues surrounding the management of antibiotics are complex because there are more than 150 compounds used as antibiotics today.

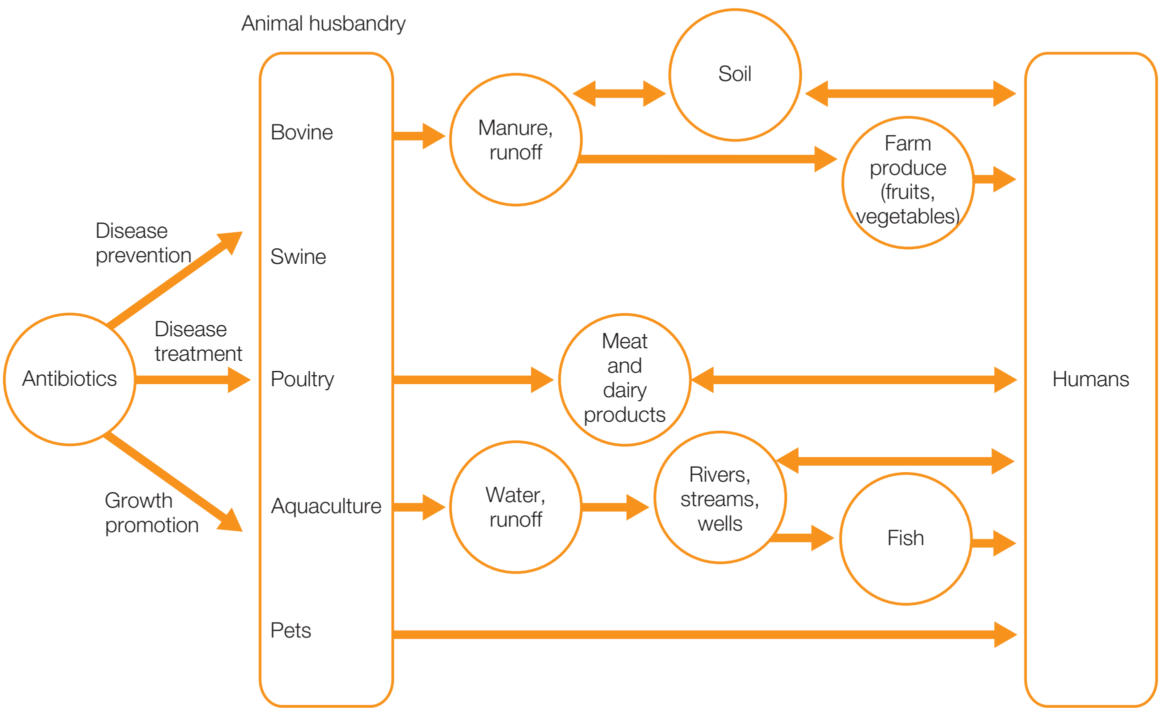


Figure 8.30 Following three kinds of antibiotic use in animals, antibiotics and antibiotic-resistant bacteria enter many habitats, and can eventually come in contact with and impact people. (Adapted from Levy, 2002.)

These compounds are partitioned between therapeutic, prophylactic, growth promotion, human, and animal usage. Figure 8.30 provides an insightful overview of the many industrial, economic, behavioral, and environmental connections between antibiotic use and human wellbeing. Looking forward, there is an obvious need to reassess personal and public policies that influence the effectiveness of antibiotics used to cure and prevent infectious disease.

What do antibiotics REALLY do for bacteria?

The above discussion has as its focal point the *human* use of antibiotics. These medically crucial compounds are produced by microorganism via biosynthetic pathways expressed in petri dishes and industrial fermentation vessels. The genes and their evolution are undeniable facts!

However, omitted entirely above is the issue of the "true" role of antibiotic compounds in the lives and ecologies of microorganisms. Selman Waksman, discoverer of streptomycin, the first useful antibiotic produced by a bacterium, has stated "antibiotics play no real part in modifying or influencing living processes that occur in nature" (Waksman, 1961). The reasoning behind Waksman's statement is that in the carbon-limited soil

environment, microbial populations are unlikely to activate the biosynthetic machinery to create and release high concentrations of antibiotic compounds. Indeed, to the author's knowledge, naturally occurring antibiotics have never been detected in soils.

Rather than presuming that naturally occurring microbial communities are constantly engaged in "microbial genocide", Julian Davies and colleagues have advocated for a more sensible paradigm (Davies et al., 2006; Yim et al., 2007; Davies and Davies, 2010; Martinez, 2008). Why not view naturally occurring communities as dwelling in a relatively stable state? Antibiotic compounds may play important roles in living processes that *maintain* that stability, rather than disrupt it. This view acknowledges that though microbial communities in nature are in flux (due to changes in the environment and available nutrients), constant warfare may not be sustainable.

Figure 8.31 provides a foundation for the role of antibiotics *not* as growth-inhibiting agents, but *instead as signaling molecules*, which may operate to maintain metabolic stability of communities. The trends in Figure 8.31 show the nature and extent of gene-activation (transcription) responses to antibiotics. Note that antibiotics (horizontal axis in Figure 8.31) trigger transcriptional responses in test bacterial species at low concentrations likely to be environmentally relevant. The signaling responses occur at concentrations that are orders of magnitude below minimum inhibitory concentrations ("MIC" in Figure 8.31) required for antibiotics to damage susceptible populations. Note also that cell growth ("multiplication" in Figure 8.31) is only impaired at very high concentrations – those at and above the MIC. The signaling (or gene regulation) role of antibiotics at concentrations below those needed for the inhibition of cell growth has surfaced in recent years. Almost all antibiotics tested to date exhibit the regulatory trends shown in Figure 8.31 (Davies et al., 2006; Yim et al., 2007). This suggests that antibiotic compounds likely participate in signaling networks that foster chemical communication between microbial cells. Intracellular receptors for the signals are almost certainly cytoplasmic macromolecular structures, such as ribosomes, the DNA replication apparatus, RNA replication apparatus, and cell-wall synthesis complexes. Note that many of these receptors have previously been identified as the inhibitory targets for antibiotics (Figure 8.28). It has been suggested (Davies et al., 2006; Yim et al., 2007; Davies and Davies, 2010) that binding of the signal molecules to their receptors at low concentrations initiates a variety of gene transcription patterns, depending on the nature of the signal molecule and the target. Modulation of host transcription likely leads to metabolic and behavioral changes in the microorganisms and their metabolism in mixed microbial communities.

Thus, the true, evolutionary roles of antibiotics at ecologically realistic concentrations (sub-inhibitory), seem to involve cell–cell signaling and transcriptional regulation of genes. The implication of these observations is that the high-concentration, medical applications of antibiotics may be fortuitous … *extremely fortunate* for the human beneficiaries of these compounds that save millions of lives daily.

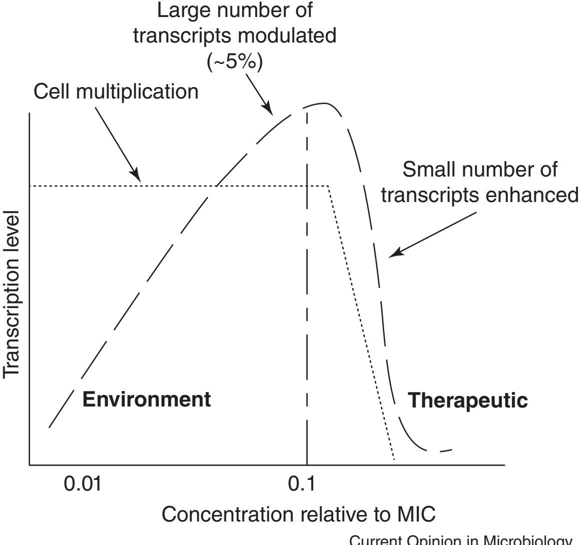


Figure 8.31 Transcriptional responses of bacteria to antibiotics are dependent on concentration. The low concentration effects are considered to be environmentally relevant, while the high (therapeutic) concentrations are likely to occur only in a medical setting. The figure shows the changes in global gene transcription related to growth inhibition (cell multiplication) as antibiotic concentration increases from subinhibitory to inhibitory (at or above the MIC, the minimum inhibitory concentration). (Reprinted from *Curr. Opin. Microbiol* **9**:445–453. Davies, J., G.B. Spiegelmana, and G. Yim. 2006. The world of subinhibitory antibiotic concentrations with permission from Elsevier.)

STUDY QUESTIONS

1 Imagine that for 10 weeks in a row, at 1-week intervals, you have scraped a film of microorganism from between your front teeth. After dispersing and plating the cells, 10 types of microbial colonies consistently grow up from agar plates with starch as the carbon source. For the same 10 weeks, you also sampled the food you eat and assembled cultured types of microorganisms from your food sources.
   (A) Outline an experimental plan aimed at distinguishing autochthonous (stable) residents of your mouth versus allochthonous (transient) residents.
   (B) Consider attempting to classify your teeth-inhabiting microflora according to the eight ecological relationships described in Box 8.1 and Figure 8.2. How difficult would it be to characterize just one of your isolated microorganisms according to the eight categories of ecological relationships shown?
   (C) Briefly list and/or describe at least four types of experimental results and/or procedures that would allow you to define the organisms as “commensals”.
   (D) List four methodological challenges that you see in the human mouth microbial system that hamper you from achieving your goal of rigorously deciding if an isolated bacterium is autochthonous, allochthonous, or a commensal.

2 If you suspect that some of the isolates from your teeth (from question 1) are antagonistic to one another:
   (A) How would you demonstrate this in the laboratory?
   (B) How would you demonstrate this in your mouth?
   (C) Would amensalism have implications for dentistry and tooth decay?
3 Information in Box 8.3 states that the microbial residents of the human intestine are "symbiotic".
   (A) Do you find the arguments convincing?
   (B) Develop counterarguments that the organisms are commensals, not symbionts.
   (C) Experimentally, what do you need to do to prove a symbiotic relationship?
   (D) Why is the proof particularly challenging for the human ecosystem?
   (E) Would it be easier to identify symbionts in other ecosystems? Why?
4 Table 8.1 lists 17 different types of symbiotic relationships.
   (A) Which of these had you never heard about prior to reading the table entries?
   (B) Write a 2–3-page essay that compares two different symbiotic relationships. One of the relationships should be chosen from Table 8.1. The second should be chosen from another source such as the peer-reviewed published scientific literature or the world wide web. For the two symbioses, organize your essay around a new table prepared as follows: make three columns labeled "characteristics", "symbiosis 1", and "symbiosis 2". Under the "characteristics" heading, enter "habitat where relationship evolved", then "key nutritional and physiological features of the habitat", then "benefits derived by the cooperating partners", then "likely sequential developments in the symbiosis, beginning with neutralism", then "physiological and/or adaptive traits achieved through symbiosis", and then "known genetic adaptations by either partner". Next fill in most (if not all) of the details of the six characteristics for both symbioses that you have chosen. The text portion of the essay should describe patterns of contrasts in the table and critically evaluate why some of the information may be particularly strong, weak, or absent.
5 Early discoveries about the mycorrhizal symbiosis were made by plant pathologists. These experimentalists routinely grow plants in sterile and nonsterile potting soil with and without a fungal inoculum. In normal assays of pathogenesis, stunted plant growth occurs in the experimental treatments that receive the fungal inoculum.
   (A) In assays with mycorrhizal fungi, what types of plant responses were found?
   (B) With the (initially) unexpected results in your answer to part A, what do you guess were the experimenter's first reactions?
   (C) What would the next likely steps be for the experimenter?
   (D) After verification, what would the next series of scientific procedures be? (Hint: think "hypotheses".)
   (E) Given the complexity of the rhizosphere habitat (Section 8.2), do you think that progress in explaining the benefits of mycorrhizal infection was easy or not? Please explain.
6 Section 8.2 presents a perspective on humans as habitats for microorganisms.
   (A) Define the "human microbiome".
   (B) What is the habitat turnover time for the skin? For the intestine?
   (C) What are the physiological and ecological implications of turnover time for the autochthonous populations? For the allochthonous populations?
7 Choose an organic compound – preferably one whose key metabolic pathway has been previously characterized in model bacteria. Be sure that the involved enzymes and genes have also been characterized. Envision this compound as a pollutant in a groundwater plume moving toward a river that provides drinking water to your home town. Reconsider what you know about the fundamentals of microbiology, physiology, biochemistry, and genetics and the many

tools used for exploring these (e.g., Chapter 6 and Chapter 7, particularly Section 7.5). Now, develop a proposal that designs and implements a *comprehensive* set of assays that can be applied to the contaminated field site. The goal of these assays should be to verify that the compound you are interested in is, in fact, being biodegraded. Please incorporate within your proposal experimental designs that include control treatments and sampling procedures (e.g., Section 6.5). State your rationale, goals, and the criteria you will be using to prove that the biodegradation reactions you hope are occurring, are truly occurring.

8 Consider microbial metabolism of tetrachloroethene.
   (A) Draw the chemical structure for the molecule.
   (B) What is the oxidation state of carbon in the molecule?
   (C) Can the carbon be oxidized?
   (D) Can the carbon be reduced?
   (E) Reason from basic principles and what the likely bioremediation strategy is for tetrachloroethene-contaminated groundwater.
   (F) Use the logic of chemistry to prepare the stoichiometric reaction for the tetrachloroethene biodegradation. Verify this by searching the world wide web and/or peer-reviewed scientific literature.
   (G) What is the electron donor in the reaction? Where does it come from in tetrachloroethene-contaminated field sites?

9 Consider microbial metabolism and bioremediation of one of the BTEX components, benzene.
   (A) Use the world wide web or other sources to find benzene's aqueous solubility.
   (B) Assume that benzene is contaminating aerobic groundwaters adjacent to a gas station and that aerobic microorganisms capable of growing on benzene are present. The rudiments of the metabolic pathway for metabolism of aromatic compounds were presented in Section 7.3 and Figure 7.11. Write out the stoichiometry of aerobic conversion of benzene to carbon dioxide.
   (C) Based on your answer to part B, how many moles of oxygen are required to biodegrade the benzene in 1 liter of benzene-saturated water?
   (D) Now recall that spilled gasoline can occur as nonaqueous-phase liquids (NAPLs) that float on the water table adjacent to spill sites. The NAPL provides a benzene supply that constantly replenishes the dissolved-phase benzene. How likely is it that the water will remain aerobic?
   (E) Explain the factors that control if and when anaerobic conditions develop in the contaminated site.
   (F) If oxygen is depleted, what happens to the benzene and other BTEX compounds?

10 Consider the bioremediation of a toxic inorganic element of your choice, say uranium.
   (A) Define the predominant chemical species for that element and their solubility, mobility, oxidation states, and the key microbiological reactions that convert one to another.
   (B) Define the microbial process(es) that attenuates the compound (e.g., attenuation is often achieved by converting the element to a form that precipitates – becoming immobile).
   (C) Once "immobilized", what steps need to be taken to be sure the element remains in place?
   (D) Can you envision any additional physical, chemical, mixing, or other engineering concerns that might hamper successful implementation of bioremediation schemes for the toxic element of interest?

11 Consider Section 8.4 on biofilms.
   (A) Had you been aware of biofilms before reading the section?
   (B) How widespread do you think biofilms are in nature? Justify your answer using ecological/environmental facts and the logic of microbial physiology.

12 DDT is an insecticide used globally from ~1934 to 1972. Despite DDT's extreme effectiveness in curbing malaria and other tropical diseases, the use of the compound was severely curtailed because of DDT's tendency to biomagnify up the ecological food chain and impair reproduction in a variety of bird species. Consider Section 8.5 on the evolution of metabolic pathways.
   (A) What is the chemical structure of DDT? (To answer this, go to the peer-reviewed literature or the world wide web.)
   (B) Given the information in Tables 8.8, 8.9, and Box 8.10 venture a hypothesis that may explain why a catabolic pathway for DDT has not yet evolved in microorganisms.

13 Consider Section 8.6 on environmental biotechnology.
   (A) Scrutinize Figure 8.16. Name two or three additional issues, concerns, and/or technologies that you feel also should be represented in the figures. Please justify your additions.
   (B) Nine case studies were presented in Section 8.6. How many of the presented cases were new to you? Choose one of the topics you had never heard of before and another (from current news stories, the peer-reviewed scientific literature, or from the world wide web) and write an essay (2–3 pages in length) comparing the histories, technical backgrounds, and prospects for technological success of the two selected studies.

14 Consider Section 8.7 on antibiotic resistance.
   (A) Which of the diseases listed in Table 8.10 have you or a family member had?
   (B) Were you and your family members cured with the help of antibiotics?
   (C) Each time you took antibiotics did you *precisely* follow the dosage guidelines?
   (D) Do you feel uneasy if you visit the doctor for an infection and he/she fails to prescribe antibiotics?
   (E) Prior to reading Section 8.7, were you aware of the extent of antibiotic use in medicine and industry?
   (F) What is the "equation" that ensures that the mechanism of evolution to antibiotic resistance continues to successfully operate and erode our shield against antibiotics?
   (G) What is the title of S.B. Levy's 2002 book about antibiotic resistance? Briefly summarize: (i) why "paradox" was chosen as a key word in the title and (ii) the current thinking on the genetic mechanisms that allow antibiotic resistance to evolve and be transferred between microorganisms.

15 Consider the far-ranging implications of the message from the final subsection in this chapter about the "true" role of antibiotics as signaling molecules. If antibiotics never reach inhibitory concentrations in natural habitats, why do microorganisms carry genes encoding antibiotic resistance? (How did the "resistome" evolve?) Is there a clear explanation out of this dilemma?

   Which of the roles for antibiotics presented (warfare agents versus signal molecules) do you feel makes the most sense? Explain your reasoning.

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9

Future Frontiers in Environmental Microbiology

Like beauty, humor, and gemstones, environmental microbiology is multifaceted.

- *Is it microbial ecology or environmental science?*
- *Is it physiology or civil engineering?*
- *Is it natural history or ecosystem science?*
- *Is it medical epidemiology or habitat colonization?*
- *Is it limnology or biogeochemistry?*
- *Is it genomics or biotechnology?*
- *Is it biodegradation or evolution?*

The answer, of course, is that environmental microbiology is all these and more. Clearly, the boundaries of environmental microbiology are blurry. Thus, a truly comprehensive text on environmental microbiology is very difficult to assemble. This present book could be 10 times its current size and still be considered incomplete. Chapters 1 and 2 of this text set the stage or background for Chapters 3 through 7, which established environmental microbiology's core set of principles, facts, logic, and methodologies. Chapter 8 provided a sampling of environmental microbiology's extensions and applications. The goal of this chapter is to succinctly examine some of environmental microbiology's potential future directions and frontiers.

Chapter 9 Outline

9.1 The influence of systems biology on environmental microbiology
9.2 Ecological niches and their genetic basis
9.3 Concepts help define future progress in environmental microbiology

9.1 THE INFLUENCE OF SYSTEMS BIOLOGY ON ENVIRONMENTAL MICROBIOLOGY

The growing discipline of systems biology will inevitably influence biology as a whole, including human medicine and environmental microbiology. Systems biology is

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the logical "next step" in the ongoing "omics" revolution. According to Kitano (2002), "to understand biology at the systems level, we must examine the structure and dynamics of cellular and organismal function, rather than the characteristics of the isolated parts of a cell or organism". The goal of systems biology is to combine molecular information of various types in models that describe and predict function at the cellular, tissue, organ, and even whole organism level. Systems biology is further defined in Box 9.1. Systems biology follows directly from the nested sciences of genomics, transcriptomics, proteomics, and metabolomics (see Sections 3.2, 6.9, 6.10, and 6.11). Now that we have ways to generate vast data sets, we need computational tools to receive and process the data (on DNA, mRNA, proteins, and metabolites) so that the data can be interpreted. Data, alone, have no meaning. Data must be converted into information.

Box 9.1

What is systems biology?

Systems biology is a way to make sense of the information flooding in from the "omics"-based procedures in biology. The four main "omics" approaches appear in the table below.

Omics system	Information
Genome: the genetic code (DNA) of an organism; determined by DNA sequencing	Genetic blueprint for cells and organisms Genome sizes range from <200,000 bp (intracellular bacterial endosymbionts) to ~3,000,000,000 bp (humans) to 149,000,000,000 bp (the Japanese flower, *Paris japonica*) The genes encode structural proteins and regulatory networks for all cellular functions (e.g., the 25 clusters of orthologous groups (COG) categories described in Section 3.2 and Table 3.2)
Transcriptome: the mRNA pool of cells, tissues, or an organism; determined by RNAseq or microarray analyses; mapped on to the genome of an organism	The subset of the genome expressed as mRNA The transcriptome is dynamic in time – responding to factors in an organism's environment that range from nutrients to organism age to disease onset
Proteome: the protein pool of cells, tissues, or an organism; determined by mass spectrometric analysis of proteins extracted and digested from whole cells; mapped on to the genome of an organism	Abundances of individual proteins in cell extracts or organ tissues at a given time under specified conditions
Metabolome: the metabolite pool of cells, tissues, or an organism; determined by liquid chromatography/mass spectrometry; mapped on to the proteome, transcriptome, and genome of an organism	Abundances of biochemical intermediary metabolites in cell extracts or organ tissues at a given time under specific conditions

When "omics" procedures are applied to a given organism (e.g., a bacterium or a human), under particular conditions, at a given time, enormous amounts of data are generated. The best way to digest, assimilate, and interpret the web-like mechanistic linkages in "omics"-based data is to use computational models (Figure 1; Kitano, 2002; Price et al., 2004; Kell et al., 2005; Arnaud, 2006; VerBerkmoes et al., 2009; Zengler and Palsson, 2012; Karsenti, 2012; see also Section 6.10). Such models of "omics"-derived data are currently research tools used to advance fundamental understanding of cell function. The integrative systems biology approach to fundamental biological questions is predicted to have applications in personalized human medicine in the future.

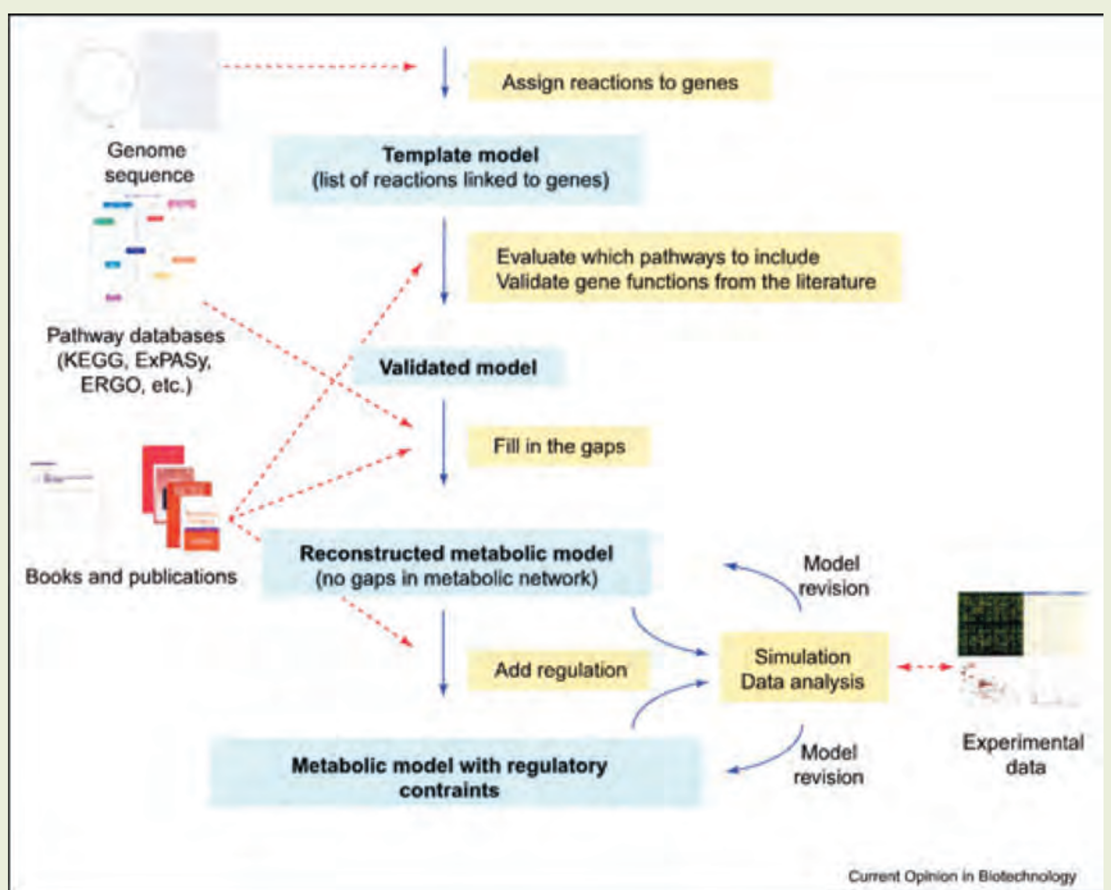


Figure 1 The process of systems biology-based metabolic model construction. Information flow is depicted as red dashed arrows and actions as blue arrows. (Reprinted from *Curr. Opin. Biotechnol.* **16**:350–355. Borodina, I. and J. Nielsen. 2005. From genomes to *in silico* cells via metabolic networks. Copyright 2005, with permission from Elsevier.)

In Chapter 3 (Section 3.2), the rudiments of genomic analyses were presented. Genomic technology cascades into the transcriptome, proteome, and metabolome. These amount to pieces of information about an organism that can be systematically assembled into hypothetical "roadmaps" of biochemical reactions and gene regulatory networks. The roadmap is a first step. Systems biology seeks "to know the traffic patterns, why such traffic patterns emerge, and how we can control them" (Kitano, 2002).

- **Where does systems biology fit in the discipline of environmental microbiology?**
Answer: Systems biology replaces "biomarkers" as one of the four basic environmental microbiological tools. Figure 9.1 presents a model of environmental microbiology in which systems biology appears instead of "biomarkers".

The ever increasing sophistication of systems biology is likely to contribute significantly toward future scientific progress in environmental microbiology. Of course, methodological improvements in microscopy, physiological incubations, cultivation procedures (Figure 9.1), and ecological theory (Prosser et al., 2007) will also contribute to future progress.

- ***When* and *where* will systems biology first be applied in environmental microbiology?**
Answer: Very soon, in simple habitats with low microbial genetic diversity.

There are two prerequisites that pave the way for systems biology approaches: (i) a relatively simple biotic system (e.g., a single organism) and (ii) a genomic map for the system. In applying systems biology to environmental microbiology, early headway can best be made using the simplest of naturally occurring microbial communities and by relying upon metagenomics as a genetic and functional blueprint for the system (see Sections 6.9

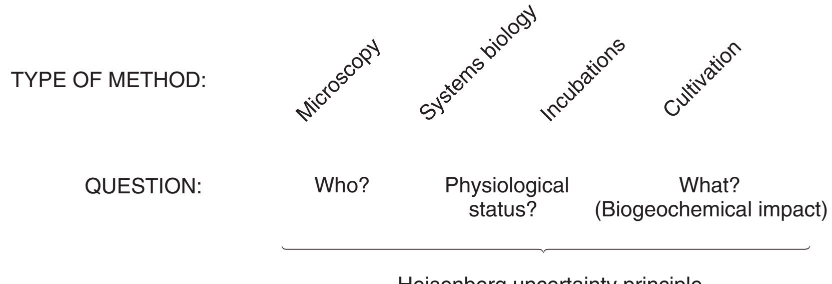


Figure 9.1 Future model of environmental microbiology. "Biomarkers" in Figure 6.5 have been replaced by "systems biology".

and 6.10). Of all the microbial systems examined to date, the one that is perhaps most environmentally extreme, and hence microbiologically simple, is the Iron Mountain acid mine site in California (Tyson et al., 2004; Lo et al., 2007; Denef et al., 2010; Denef and Banfield, 2012). As mentioned in Section 6.9 (Table 6.7), this site features a pH of <1, very low nutrient status, and very low microbial diversity; both metagenomic and proteomic analyses have led to extensive pioneering insights into both community function (Ram et al., 2005; Allen et al., 2007; Denef et al., 2010) and in situ evolution (Denef and Banfield, 2012). Thus, the Iron Mountain microbial community and habitat constitute an excellent prototype study for using systems biology to advance environmental microbiology.

Another ideal prototype system for applying systems biology to environmental microbiology is the ocean. Marine microbial ecologists have an immense advantage over other microbial ecologists because their study habitats (ocean waters) are: (i) globally distributed; (ii) well mixed; (iii) accessible at a human scale; (iv) amenable to thorough geochemical habitat characterization methods; and (v) populated by relatively low-density, low-diversity microbial communities. In contrast to marine microbiology, many other subdisciplines in environmental microbiology (e.g., soil and sediment microbiology) focus on a habitat with immense spatial heterogeneity at micrometer and other scales – where microbial inhabitants may never be fully sampled and where the environmental resources that exert selective pressures have never been fully characterized (see Section 4.6). Marine microbiologists can sample and map geochemical gradients that occur over tens of meters. While spatially discrete microhabitats and nutritional gradients do occur in ocean waters (Stocker, 2012), compared to soils, marine water columns are relatively uniform. Moreover, sampling does not leave a hole behind in the habitat. The sampled waters can be processed using flow cytometers that are capable of detecting *all* the cells in the sample. Furthermore, laser-light interrogation of biomarkers carried by the native microbial cells can often distinguish and quantify previously defined, fully sequenced, genomic "ecotypes" of microorganisms native to the ocean and known to function there (such as *Prochlorococcus*, *Pelagibacter*, *Silicibacter*, *Alteromonas*, and *Trichodesmium*). The combination of habitat characterization and genomic tools allow basic ecological questions about selective pressures and adaptations to be posed. Hypotheses naturally follow. Issues such as mechanisms controlling biogeographic patterns (growth, nutrients, other resources, predation, viruses, adverse geochemistry, competition, light, etc.) can be addressed. Particularly insightful in the development of mechanistic hypotheses linking native microbial communities to habitat characteristics have been the "ocean time series" investigations (Fuhrman 2009; Giovannoni and Vergin, 2012; Ducklow et al., 2009; Fuhrman et al., 2006; Karl et al., 2012; Ottesen et al., 2013; see also Section 6.8).

Box 9.2 describes a landmark early example of a genomics-assisted investigation: "ecological genomics" in marine microbiology. Ecological genomics is an important step towards using systems biology in environmental

Box 9.2

Moving toward systems biology: an early landmark investigation in ecological genomics of the ocean (Coleman et al., 2006)

Background

- *Prochloroccus* is a numerically dominant, photosynthetic bacterium (Figure 1) in oligotrophic ocean waters – sometimes accounting for up to half of the photosynthetic biomass of some regions.
- The ocean-water habitat of *Prochlorococcus* presents gradients of light, temperature, and nutrients.
- Several strains of *Prochlorococcus* have been isolated and cultured in the laboratory and their genomes have been sequenced. Thus, a genetic blueprint for these organisms has been established.

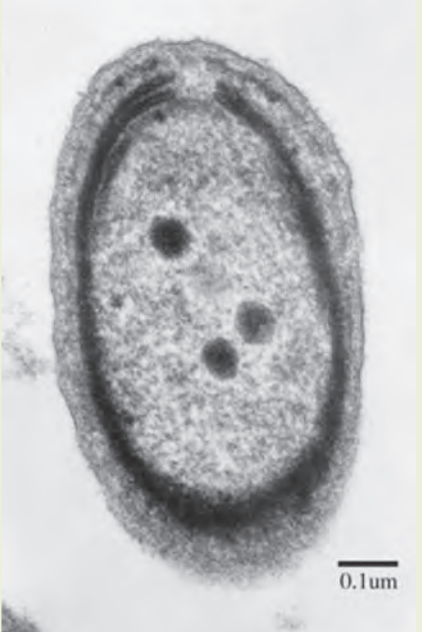


Figure 1 Photomicrograph of *Prochlorococcus*. (Courtesy of Claire Ting, Department of Biology, Williams College, with permission.)

What Coleman et al. (2006) did

- The genome sequences of two different strains of *Prochlorococcus* were compared and five large pieces of foreign DNA, termed "genomic islands", were discovered. Based on a variety of criteria, the foreign pieces of DNA did not resemble the DNA of the host – they stood out like islands surrounded by water (see Chapter 5, Science and the citizen box). "Signatures of mobility" in the DNA sequences indicated that viruses were the agents that imported the foreign DNA.
- By analyzing the sequences of genes encoded in the genomic islands, Coleman et al. (2006) were able to discern that the islands conferred specific physiological functions such as nutrient uptake (amino acids, manganese, and cyanate), stress response (to light and low phosphate), and susceptibility to virus infection (via cell surface molecules).
- In laboratory assays of gene expression under high- and low-light conditions, a differential expression of genomic island genes was observed. This confirmed their physiological importance.
- Genomic fragments from the naturally occurring microbial community were retrieved from the Sargasso Sea and from Hawaiian waters. Many of these genomic fragments contained gene sequences that could be mapped on to genomic island-containing regions of *Prochlorococcus* chromosomes in the cultivated strains.
- Clear patterns in variations of the genomic islands in wild populations emerged.

What the findings mean

The genomes of wild populations of *Prochlorococcus* seem to be composed of a consistent "core", augmented by islands of high genetic variability. Functional genes carried within the islands

play an important role in allowing distinctive ecotypes of *Prochlorococcus* to adapt to selective pressures such as high-light conditions in shallow waters and low-nutrient conditions. Via these genomic islands, different niches in the oceans seem to be exploited by distinctive *Prochlorococcus* populations.

The future

An inquiry has recently been completed that examined hundreds of wild, uncultured *Procholococcus* cells residing in the Atlantic Ocean near Bermuda Island (Kashtan et al., 2014). Procedures utilizing a combination of flow cytometry, single-cell genomics, and the rRNA gene's highly variable intergenic transcribed spacer (ITS) region were utilized. Results revealed that metabolic fitness in *Prochlorococcus* may arise from exchange of functional gene cassettes between hundreds of diverse subpopulations defined by variable genomic backbones.

microbiology. As described in Box 9.2, Coleman et al. (2006) had developed the right tools (e.g., ocean waters as habitat, *Prochlorococcus* as a key photosynthetic player in the habitat, cultivated representatives of *Prochlorococcus* with sequenced genomes, metagenomic techniques for community DNA, and distinctive physiological characteristics of *Prochlorococcus* strains from shallow and deep water) for asking basic questions about evolution. The report by Coleman et al. (2006) made a convincing and insightful case for *Prochlorococcus* adaptation and niche differentiation (see Section 9.2) caused by "genomic islands" mobilized by viruses, within naturally occurring *Prochlorococcus* populations.

To emphasize the promise of marine microbial habitat for systems biology, it should be recognized that E. DeLong and D. Karl (DeLong and Karl, 2005; Karl et al., 2012; Ottesen et al., 2013) have established an international center for microbial oceanography. A systematic plan is being implemented that links activities and information from a dozen oceanography-related subdisciplines (Figure 9.2). Note that core activities at the heart of Figure 9.2 include genome libraries, bioinformatics, DNA arrays, proteomics (see Sections 6.9, 6.10, and Table 6.4), and modeling – all reflecting influences of systems biology.

Finally, Zengler and Palsson (2012) have recently coined the term "community systems biology" as a means by which systems biology can merge with and advance environmental microbiology. Modeling entire microbial communities in silico to predict their behavior is a key goal of the emerging discipline of community systems biology (Zengler and Palsson, 2012). Such an achievement would represent the long-sought milestone of comprehensively understanding microorganisms and their interactions with both other species and with their environment. The strategies listed in Box 9.3 describe the progression that is likely to occur as systems biology tools gain sophistication while simultaneously embracing environmental microbiology. To date, systems biology is only a reality

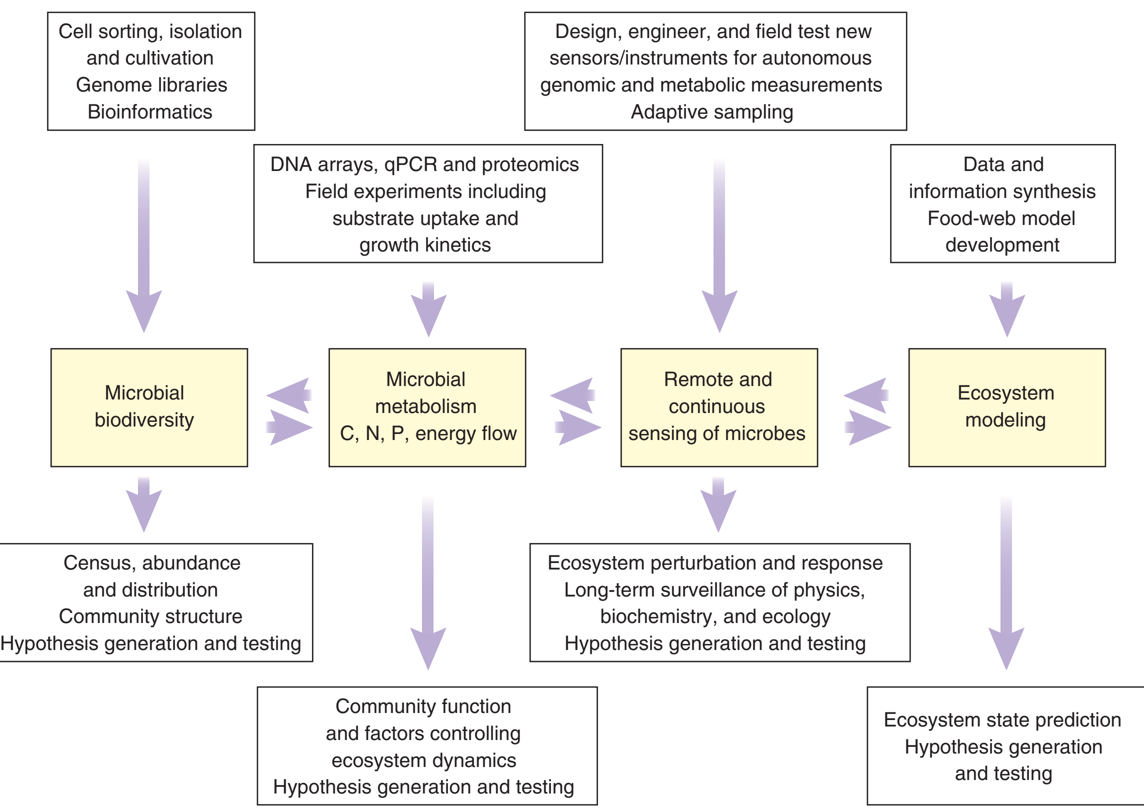


Figure 9.2 Organizational plan for coordinating the dozen subdisciplines necessary to advance understanding of microbial oceanography. QPCR, quantitative polymerase chain reaction. (Reprinted by permission from Macmillan Publishers Ltd: *Nature*, from DeLong, E.F. and D.M. Karl. 2005. Genomic perspectives in microbial oceanography. *Nature* **437**:336–342. Copyright 2005.)

for single-genome microbial systems (entry 1, Box 9.3). Computational modeling pipelines currently are successful at using principles of microbial biochemistry, physiology, and bioinformatics (and other disciplines) to predict how cellular networks interact to create a microorganism's emergent phenotype from its genome. According to Zengler and Palsson (2012), an analogous path forward to community systems biology will build through a series of increasingly complex stages (Box 9.3). Already progress is being made at modeling metabolic interactions between known mixtures of microbial populations with known genomes. The next step (3 in Box 9.3) is to develop functional models of interacting networks of elements (environmental parameters, population surveys, metagenomes, metatranscriptomes, metaproteomes) derived from functioning microbial communities in their habitats. The final goal of successful, accurate quantitative modeling of microbial ecosystems and their biogeochemical characteristics awaits future advancements in omics, bioinformatics, geochemistry, and modeling (entry 4, Box 9.3).

Box 9.3

Progressive application of systems biology to build integrative models aimed at explaining how complex behaviors emerge from interactions of populations comprising microbial communities in natural habitats (Zengler and Palsson, 2012; Karsenti, 2012)

Approach	Strategy
1. Systems biology of a pure culture	• Use genome sequence, metabolic pathways, chemical reactions, growth yields, cell biosynthetic pathways, respiratory pathways, etc., to model and predict the phenotype of single microbial cell. STATUS: Established automated pipeline now predicts phenotype from genomes.
2. Systems biology for known cell mixtures (simple communities)	• Use *multiple* genome sequences and their respective metabolic pathways, chemical reactions, growth yields, cell biosynthetic pathways, respiratory pathways, etc., to model and predict the "community" phenotype for a limited number of interacting microbial populations. STATUS: Moderate success in current models at predicting metabolic *interactions between* cells whose genomes are sequenced.
3. Systems biology-based correlational analyses	• Use an integration of omics procedures carried out on naturally occurring microbial communities. Correlations between habitat ecological parameters and community members, their expressed genes, and their proteins can lead to hypotheses about cell–cell interactions and roles of key player in the system. STATUS: Even with state-of-the-art computational methods, correlation cannot establish causality, but valuable hypotheses are generated.
4. Systems biology of microbial ecosystems	• Use quantitative models to predict the current and future status of microbial ecosystems based upon data generated from many omics procedures carried out on naturally occurring microbial communities. STATUS: Awaiting future advancements in omics, bioinformatics, geochemistry, and modeling.

9.2 ECOLOGICAL NICHES AND THEIR GENETIC BASIS

Genome as evolutionary record

It is a truism that the current state of a microorganism's genome reflects its heritage. That heritage is a series of evolutionary events that combine vertical transmission of genes, lateral gene transfer, gene loss, mutations,

rearrangements, and selective pressures (Kunin and Ouzounis, 2003; Kazazian, 2004; Ochman and Davalos, 2006). The legacy of such events, the genome itself, can be viewed as a collection of robust, highly refined genetic networks. However, mixed in with the evolutionary "successes" (the sophisticated regulatory networks) are evolutionary "failures" and "works in progress" that include random insertions, pseudogenes, and remnants of traits no longer useful (Kunin and Ouzounis, 2003; Ochman and Davalos, 2006). Thus, even in the postgenomic age, the detailed evolutionary histories of microorganisms are obscure. There is no doubt, however, that selection, ecological fitness, and niche have been dominant forces shaping the outcomes of genome evolution.

Ecological niches

It is also a truism that every organism has its own ecological niche. Niche is an idea, a concept that humans have created about the ecological role of a species in its ecological community (Ricklefs and Miller, 2000). Hutchinson (1958) originated the concepts of "niche space" and "niche hyper volume". These ideas are based on the notion that environmental factors (such as temperature, pH, nutrient availability, food types, and food size) occur as gradients that can be plotted as axes in n-dimensional space. For each environmental factor (each dimension in niche space), a species can survive over a defined range. When all of the ranges for each of n environmental factors are integrated together, they can be plotted in n-dimensional space to represent a "Hutchinsonian hypervolume" that defines niche. The *fundamental niche* (Figure 9.3) is the largest ecological hypervolume that an organism or species can possibly occupy and is based mainly on interactions with the physical environment, in the absence of competition. In contrast, the *realized niche* (Figure 9.3) is the portion of the fundamental niche that the organism occupies after interacting, especially competing, with other organisms. Competition between organisms for resources is considered a major evolutionary and ecological force. Competitive exclusion of one organism by another occurs because no two species can occupy the same niche. In nutrient-rich, stable, physically diverse habitats (such as tropical rain forests), high species diversity is thought to be the result of niche differentiation. Species evolve toward differences in niche. In so doing, competition between species is reduced (Whittaker, 1970).

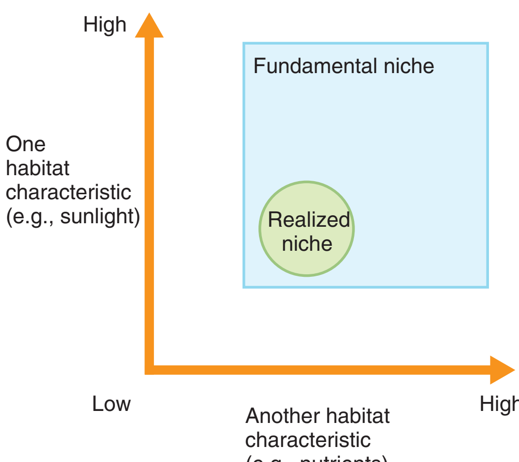


Figure 9.3 A view of fundamental and realized ecological niches in two dimensions. (Based on Hutchinson, 1958.)

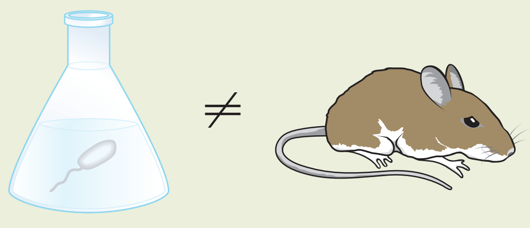
Methodological strategies for discovering the genetic basis for ecological fitness

Discovering the ecological niches of microorganisms within their native habitats is a major goal of environmental microbiology (See Sections 6.10 and 6.11). *An even more fundamental issue is discovering the genetic basis for ecological fitness and niche*. This area represents a major frontier for future research (Rediers et al., 2005; Saleh-Lakha et al., 2005; van Dillewijn, 2008).

In medical microbiology, a variety of elegant molecular biological strategies have been employed to discover the genetic basis for infectious diseases (Box 9.4). Disease prevention strategies rely upon a mechanistic understanding of biochemical and signaling pathways in pathogenic microorganisms and their hosts. The molecular approaches described in the top of Box 9.4 have successfully identified, in pathogenic bacteria, virulence factors that are expressed only in an infected host. There is an obvious parallel between medical microbiology and environmental microbiology: the infected host is equivalent to colonized soil, sediment, or body of water (Box 9.4, bottom). Both disciplines advance by identifying genes for ecologically important processes that are expressed differentially in habitats of interest.

Box 9.4

Discovering the genetic basis for ecological fitness



In medical microbiology

When a microorganism is grown in a laboratory flask, a small subset of its entire genome is activated (transcribed into mRNA) and manifest as the proteins that catalyze the catabolic and anabolic reactions that create new cells. Medical microbiologists have persistently sought ways to identify virulence genes that are crucial for causing diseases in humans and animals. Such genes are ordinarily intractable (unexpressed) during routine laboratory growth of bacterial pathogens. Once discovered and understood, virulence genes serve as gateways for battling diseases.

- **How can genes for pathogenesis be discovered?**

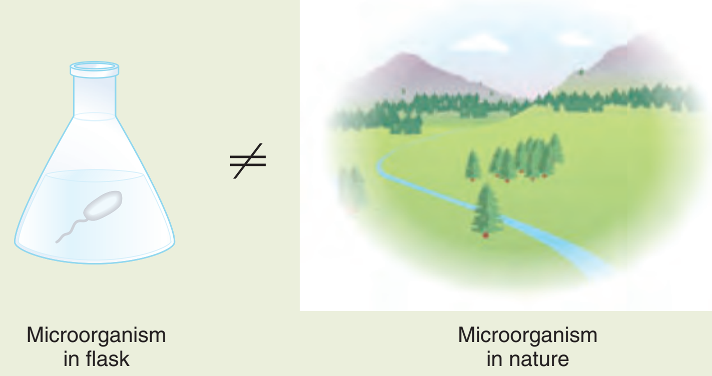
Answer: Devise a way to identify genes expressed by pathogens exclusively while they are inside a diseased host. The chances are good that these "habitat-specific" genes are necessary for disease development and/or survival in the host.

Box 9.4 *Continued*

Many different techniques to find the host-induced genes for pathogenesis have been devised. The procedures include: "*in vivo* expression technology", "differential fluorescence induction", "signature-tagged mutagenesis", "differential display using arbitrarily primed PCR", "subtractive and differential hybridization", and "selective capture of transcribed sequences" (Chiang et al., 1999; Rediers et al., 2005).

Broadly, these techniques adopt either of two basic strategies:

1. Screening mRNA pools extracted from pathogens within their hosts. Contrasts between genes expressed in the host versus in a control (nonhost) environment reveal host-specific genes that are then cloned and sequenced.
2. Screening mutant strains of a pathogen for rare genes that are induced specifically and confer survival while within the host (this involves careful creation of a large library of mutation-carrying strains, comparing their survival inside versus outside a host, and characterizing (sequencing) the genes of interest). The final validation step involves proving the mechanistic link between genotype and phenotype by restoring function after reintroduction of the wild-type version of the gene.



In environmental microbiology

Though pursued less frequently, the two broad strategies for discovering the genetic basis for ecological fitness in environmental microbiology are completely analogous to those used in medical microbiology:

1. Correlational analyses based on mRNA (metatranscriptomic) and protein (metaproteomic) pools extracted from natural habitats. Such information provides key insights into the identity of community members and their in situ metabolic functioning, especially when completed in conjunction with field site-specific biogeochemical conditions (see Sections 6.10 and 6.11). This is the correlative systems biology approach (step 3) described in Box 9.3.
2. Genetic manipulations of cultivated representatives of the naturally occurring microbial populations of interest. Due to successful cultivation efforts, model microorganisms truly representative of those active in field sites are becoming increasingly available (e.g., *Pelagibacter*, *Alteromonas*, *Geobacter*, *Polaromonas*, *Prochlorococcus*, *Desulfovibrio*). For many of these ecologically relevant cultivated microorganisms, genetic manipulation systems are in place that allow verification of the impact of genotype on phenotype via site-directed mutagenesis procedures that knock out individual genes and subsequently back-complement them (thereby restoring phenotypic function)

As described in Box 9.4, for medical microbiology there are two basic strategies for finding ecologically significant, fitness-conferring genes: (i) screening of mRNA pools extracted from the habitat of interest (versus a control habitat) and (ii) screening a large library of mutant strains for genes that are specifically induced in the native habitat and confer survival to the host. Once the gene conferring a particular pathogenic fitness trait has been found, sequencing allows its character and phylogeny to be discerned and the combination of genetic complementation and phenotypic restoration validate the mechanstice tie between genotype and phenotype.

The medical-microbiology strategy for discovering the genetic basis of ecological fitness does not apply perfectly to environmental microbiology for at least three reasons: (i) sometimes biogeochemical processes are the result of many cooperating populations; (ii) key active populations may not yet have cultivated representatives; and (iii) genetic-manipulation systems for the cultivated representatives may not yet be developed. Nonetheless, the broad logic developed by medical microbiologists still applies – strategies quite analogous to those described for medical microbiology have begun to be implemented in environmental microbiology. For example, with the aid of omics procedures, functioning naturally occurring microbial communities can be sampled and their active members, genes and proteins, can be identified (Box 9.4; see also Sections 6.10 and 6.11). Correlations between networks of biomarkers and field biogeochemical parameters can provide insights and lead to hypotheses about the in situ roles of particular microbial populations and their genes. Regarding genetic manipulations that test detailed hypotheses about the genetic basis of ecological fitness, there has been some progress. Groh et al. (2005) developed a system for using a signature-tagged mutagenesis (STM) approach on *Desulfovibrio desulfuricans* and *Shewanella oneidensis* to study genes contributing to survival in sediment. The procedure successfully identified mutants in chemotaxis genes in both test bacteria. STM-identified genes conferring fitness for the sediment habit were to be identified in a future publication. More recently, a combination of both whole genome sequencing and site-directed mutagenesis has successfully been used on a dual-culture of *Geobacter* strains to prove that a single genomic mutation in *Geobacter sulfurreducens* (in the *pilR* regulatory gene, leading to enhanced expression of multiheme c-type cytrochome that promotes electron transfer) is responsible for drastically enhancing the ability of *G. sulfurreducens* to syntrophically (cooperatively) grow under anaerobic conditions with the another *Geobacter* strain (Summers et al., 2010).

Based on information shown in Box 9.4, it is clear that there is promise for applying medical gene-discovery procedures to naturally occurring microbial communities in their habitats. The genes and proteins expressed in situ by naturally occurring microorganisms provide glimpses of the identity of active populations, their physiological status, their interactions, and the real-world resources that the populations confront.

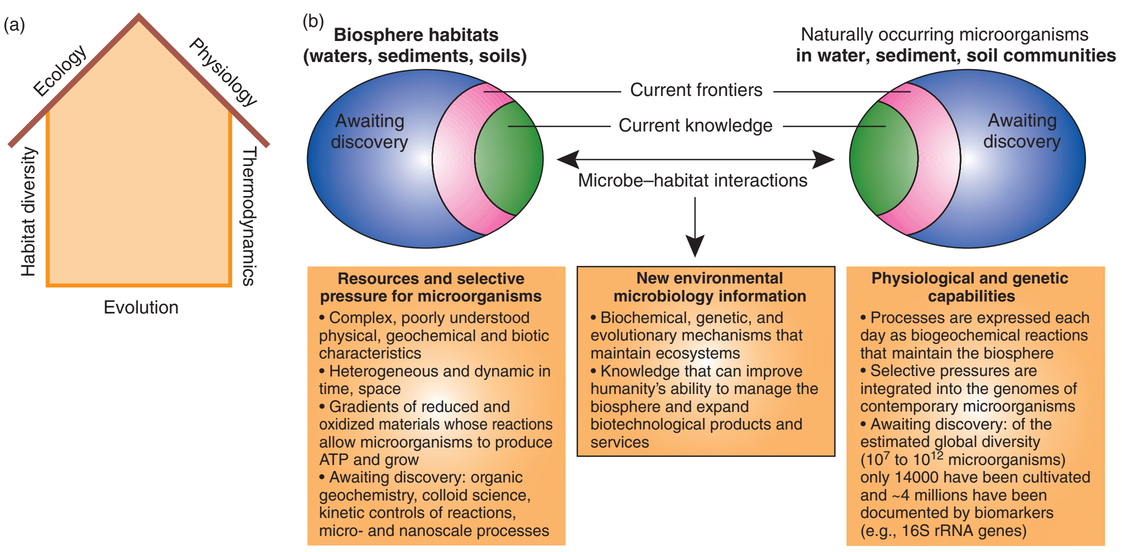
Understanding the genetic basis for niche, fitness, and ecological success is a meritorious goal and a significant challenge for future environmental microbiologists. To quote Rediers et al. (2005):

> Analysis of ecological success is far from straight-forward: it is a complex and multidimensional phenotype determined by interconnected regulatory pathways involving both individual genes and gene networks. Natural selection, which is largely responsible for shaping the determinants of ecological success, does so by operating on interacting systems (more so than on single genes) to generate specific morphologies, physiologies and behaviors.

9.3 CONCEPTS HELP DEFINE FUTURE PROGRESS IN ENVIRONMENTAL MICROBIOLOGY

Figure 9.4 provides a summary of six ways to conceptualize the essence of environmental microbiology and its goals:

1. Figure 9.4a (from Section 1.1) reminds the reader of the five basic components (evolution, habitat diversity, thermodynamics, physiology, and ecology) that build the "house of environmental microbiology".
2. Figure 9.4b (from Figure 1.5) emphasizes the dynamic interfacial nature of environmental microbiology. Frontiers emerge as progress is made simultaneously in environmental science and microbial ecology.
3. Figure 9.4c (from Figure 6.13) portrays environmental microbiology as the iterative (cyclic) application of methodological tools from many biological disciplines to field sites. The tools are applied in ways that confirm and validate the identities of environmentally relevant microorganisms and their genes.
4. Figure 9.4d (the concentric circle model) emphasizes that Chapter 6's fundamental questions (Who?, What?, When?, Where?, How?, Why?) are surrounded (enabled and limited) by the methodological tools used by environmental microbiologists.
5. Figure 9.4e (from Figure 6.5) divides the many environmental microbiological tools into four basic types (microscopy, biomarkers, incubations, and cultivation) and emphasizes two inescapable experimental facts: (i) the validity of all data generated by experimental approaches is threatened by environmental microbiology's Heinsenburg uncertainty principle (see Section 6.4) and (ii) when sampling real-world microbial habitats, each of the four types of experimental tools demands an optimal sampling procedure.
6. The message from Figure 9.4f (from Box 5.1) is that no single type of environmental microbiological tool is sufficient to deliver robust, new information. Instead, complementary information from independent, convergent sources is the path toward progress.



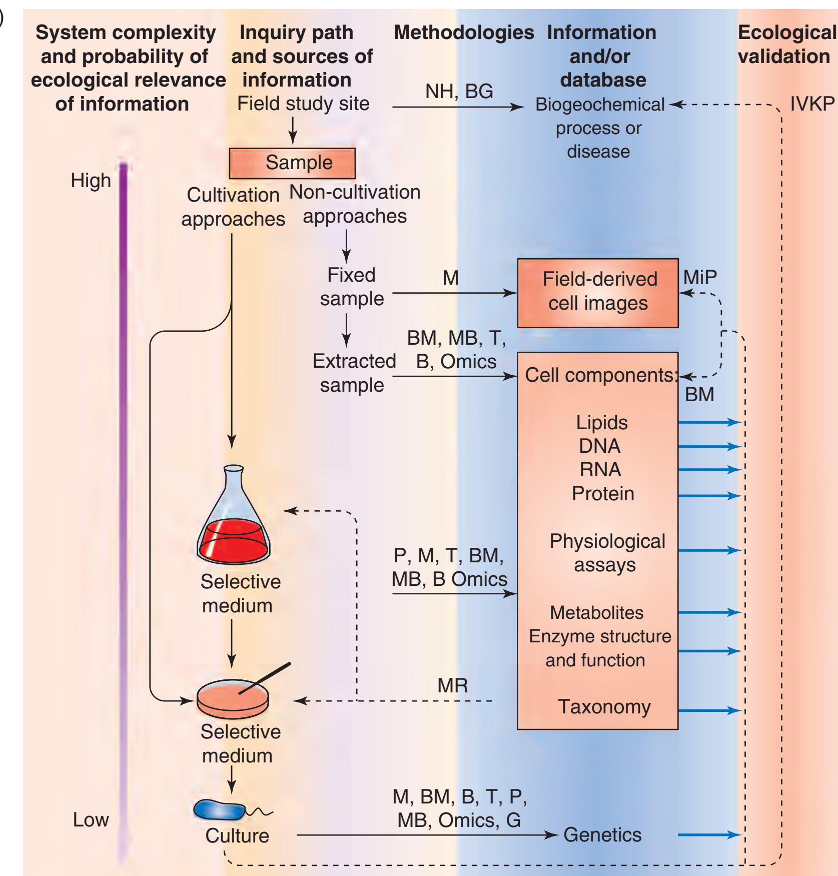


Figure 9.4 Six diagrams that conceptually define environmental microbiology. See text for details.

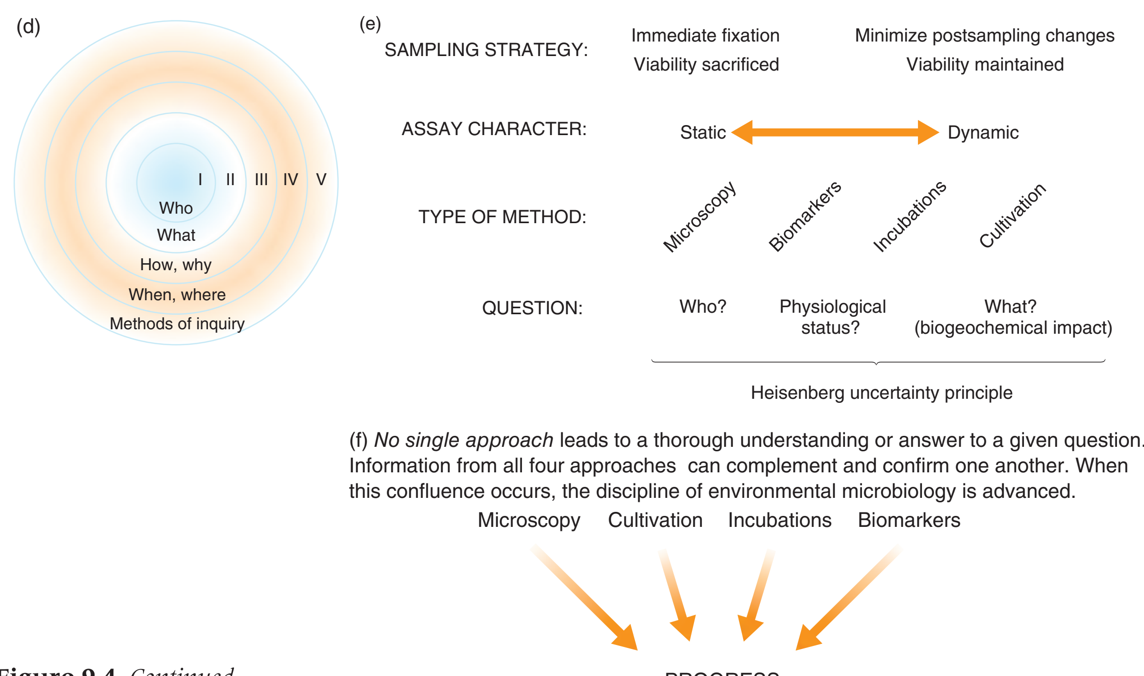


Figure 9.4 *Continued*

• This begs the question: What is progress?

Information in Box 9.5 attempts to define "scientific progress" in environmental microbiology and predicts that accelerated progress is inevitable. The metaphor for advancing the discipline as environmental microbiology is not one of a mountain that, once climbed, is conquered. Instead, a more appropriate metaphor for environmental microbiology is the human mind. Humans never acquire complete, true, and accurate knowledge of the world that surrounds them. The constant accrual of sensory and other information throughout our lifetimes allows us to navigate the world – even if the full complexity (chaos?, beauty?, reality?) of our surroundings is never fully revealed. The "mind" of environmental microbiology is an accruing synthesis of facts, principles, and relationships derived from environmental microbiology's many contributing disciplines (from microbiology and soil science, to oceanography and ecology, to physiology and biochemistry, to genomics and systems biology; see Section 1.5 and Table 1.5). We will never know it all because there is always more to discover – new habitats, new cultured microorganisms, physiological adaptations, ecological relationships, selective pressures, enzymatic structures, even new worlds (e.g., Mars). To quote C. Woese (2004):

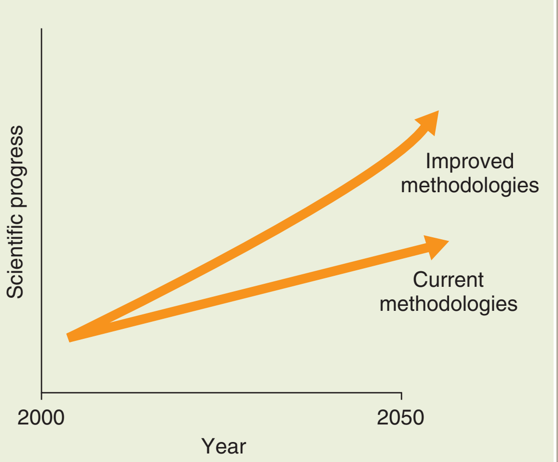
> Science is an endless search for truth. Any representation of reality we develop can be only partial. There is no finality, sometimes no single best representation. There is only deeper understanding, more revealing and

enveloping representations. Scientific advance, then, is a succession of newer representations superseding older ones, either because an older one has run its course and is no longer a reliable guide for a field or because the newer one is more powerful, encompassing, and productive than its predecessor(s).

Box 9.5

Defining progress in environmental microbiology

- Scientific progress in environmental microbiology can be defined as "adding new information to the advancing frontiers of Who?, What?, When?, Where?, How?, and Why?"
- This book is simultaneously a synopsis of, and testimony to, progress in environmental microbiology.
- The path forward in environmental microbiology is depicted by two arrows on the graph to the right.



Current methodologies (low arrow scenario)

If current methods remained static, environmental microbiologists could march onward for ~50 years describing new cultivated microorganisms (Chapters 5 and 6), discovering new uncultivated microorganisms (Chapters 5 and 6), describing new habitats, symbioses, evolutionary adaptations (Chapters 4 and 8), and their biochemical, genetic, genomic, and structural biological bases (Chapter 7). These discoveries appear weekly and/or monthly in peer-reviewed scientific journals such as *Science*, *Nature*, *Proceedings of the National Academy of Sciences*, *Applied and Environmental Microbiology*, *ISME* (International Society for Microbial Ecology) *Journal*, *Environmental Microbiology*, *Microbial Ecology*, *FEMS Microbial Ecology*, *Geomicrobiology*, and others.

Improved methodologies (high arrow scenario)

Environmental microbiology is a methods-limited, multidisciplinary science (see Chapters 1 and 6). Methodological advancements are constantly being made in the sets of tools used by environmental microbiologists (e.g., microscopy, analytical chemistry, DNA sequencing, proteomics, nanotechnology, microbial cultivation procedures, systems biology). Therefore, the rate of progress in environmental microbiology over the next several decades will certainly accelerate.

Thus, predicting the details of future progress in environmental microbiology is not facile. As shown in Box 9.5, accelerating progress is certain. This progress must be a balance between an ever-deepening understanding of environmental microbiology's many subdisciplines and the emergent properties of complex systems (Woese, 2004).

STUDY QUESTIONS

1 On systems biology:
   (A) If you were an instructor of an introductory biology course, how would you define systems biology?
   (B) What means and examples would you use to convey what systems biology seeks to accomplish?
   (C) Does it make sense to apply systems biology to environmental microbiology now?
2 On the genetic basis of fitness:
   (A) What ecological system do you find particularly fascinating? (Name one, for example, coral reef, redwood forest, salt marsh.)
   (B) Why?
   (C) What microbiologically mediated biogeochemical processes occur there? (Name several.)
   (D) Venture several guesses about specific fitness traits that microorganisms need to have to be successful in your chosen ecosystem.
   (E) How would you investigate the genetic basis of these fitness traits?
3 Which of the conceptual models for the organization of environmental microbiology (see Figure 9.4) do you find to be most insightful in encompassing the many elements of the discipline? Why?

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Glossary

ABC transporter ATP binding cassettes represent a superfamily of oligopeptide permease proteins responsible for transporting a wide range of substrates across membranes.

agar A vegetable gelatin made from various kinds of algae or seaweed. Used as a gelling agent in solid culture media for isolating and cultivating microorganisms.

agar plate An agar plate is a sterile Petri dish that contains agar plus culture media and is used to cultivate microorganisms.

algae Chiefly aquatic, eukaryotic one-celled or multicellular plants without true stems, roots, or leaves, that are typically autotrophic, photosynthetic, and contain chlorophyll.

allelochemical a chemical produced by one organism, exerting a detrimental physiological effect on the individuals of another species when released into the environment

allochthonous Transported in from elsewhere; materials found in a place other than where they and their constituents were formed.

amoeba Any of various one-celled aquatic or parasitic protozoans having no definite form and consisting of a mass of protoplasm containing one or more nuclei surrounded by a flexible outer membrane. Amoebae move by means of pseudopods.

anabolism Metabolic reactions that build cellular constituents, consuming energy (ATP) in the process.

Archaea One of the three major trunks (domains) in the tree of life based on small subunit rRNA phylogeny.

assimilation, assimilatory Physiology. The conversion of nutrients into living tissue via biosynthetic metabolic reactions.

autochthonous Originating or formed in the place where found.

ATP Adenosine triphosphate, the energy currency of cellular metabolism. Catabolism generates ATP, anabolism consumes it.

autotroph An autotroph (or primary producer) is an organism that uses CO_2 as a carbon source. CO_2 fixation is driven by energy (ATP) derived from light or chemicals.

auxin Any of several plant hormones that regulate various functions, including cell elongation.

Bacteria One of the three major trunks (domains) in the tree of life based on small subunit rRNA phylogeny.

bacteria A generic term referring to prokaryotic forms of life (*Bacteria* and *Archaea*). The use of the term "bacteria" is historical baggage from early microbiologists'

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inability to distinguish between *Bacteria* and *Archaea*. In contemporary biology, the term is inherently inaccurate and often deemed obsolete. Because *Bacteria* and *Archaea* are vastly different (phylogenetically and phenotypically), referring to these two domains with a single term may cause erroneous thought.

basalt The most common type of solidified lava; a dense, dark gray, fine-grained igneous rock that is composed chiefly of plagioclase, feldspar, and pyroxene minerals.

biomarker A biochemical substance whose presence and structure serves as a signature of life. Examples of biomarkers include 16S rRNA, membrane lipids, and chlorophyll.

catabolism The metabolic breakdown of large molecules in living organisms, with accompanying release of energy, captured as ATP.

chemosynthesis, chemosynthetic Generation of ATP by reactions that oxidize organic and/or inorganic substances.

chromatin Condensed chromosomal DNA.

chromosome Major reservoir of genetic information in a cell; haploid cells have a single copy of their chromosome, while diploid cells have two copies.

ciliate, ciliated protozoa Any of various protozoans of the class *Ciliata*, characterized by an exterior covered with numerous short, whip-like appendages, called cilia (*see* flagellum).

clade A group of organisms, such as a genus, whose members share homologous features derived from a common ancestor. On a phylogenetic tree, a clade is a coherent cluster of related sequences.

conjugation Genetic transfer of plasmid DNA between cells; cell-to-cell contact is required.

cultivated *See* cultured.

culturable (*adj.*) Capable of being cultured. A microorganism is culturable if a suitable liquid or solid medium, supporting propagation, has been devised.

cultured (*adj.*) A microorganism that can be and has been grown under defined, controlled conditions. Domesticated.

cyanobacteria Free-living prokaryotic organisms without organized chloroplasts but having chlorophyll *a* and oxygen-evolving photosynthesis; capable of fixing nitrogen in heterocysts. Occurring in lichens and other symbiotic relationships. Commonly called "blue-green algae".

detritus Disintegrated or eroded matter: the remnants of prior life, "the detritus of the past".

diagenesis Geology. All the physical, chemical, and biologic changes undergone by sediments from the time of their initial deposition, through their conversion to solid rock, and subsequently to the onset of metamorphism.

dissimilation, dissimilatory Physiology. Participation in cellular processes without incorporation into biomass. Final electron acceptors are essential for physiological reactions but their reduced forms are waste products (e.g., H_2O, H_2S, CH_4) that are not assimilated.

elective enrichment A method of isolating microorganisms capable of utilizing a specific substrate by incubating an inoculum in a medium containing the substrate. The medium may contain substances or have characteristics that inhibit the growth of unwanted microorganisms.

electron microscopy Any of a class of microscopic procedures that use electrons rather than visible light to produce magnified images.

endolithic life Literally, "within rock". Microorganisms that have colonized and live within the pore space of geologic formations, from Antartica to the ocean floor.

endospore A small asexual spore, produced within a cell, that serves as a resistant resting stage in some prokaryotes.

enrichment culture A medium of known composition, providing specific physiological conditions to favor the growth of a particular type of microorganism.

epifluorescent microscopy A microscopic technique in which the impinging short-wavelength light causes target fluorescent molecules to reflect longer wavelength light back to the viewer.

Eukarya One of the three major trunks (domains) in the tree of life based on small subunit rRNA phylogeny.

eutroph A physiological type of microorganism that is suited to growth on nutrients at high concentration.

Eutrophic (*adj.*) A habitat having waters rich in mineral and organic nutrients that promote a proliferation of microorganisms. If large blooms of photosynthetic algae occur in eutrophic water, their decay by heterotrophic prokaryotes may deplete oxygen in the water.

facultative Versatile; capable of functioning under varying environmental conditions. The term is often applied to microorganisms that can live with or without oxygen (facultative anaerobe).

flagellated protozoa Any of a large group of single-celled, microscopic, eukaryotic organisms having one or more large whip-like appendages, known as flagella (*see* flagellum)

flagellum In prokaryotes, a whip-like motility appendage present on the surface of some species. Flagella are composed of a protein called flagellin. Prokaryotes can have a single flagellum, a tuft at one pole, or multiple flagella covering the entire surface. In eukaryotes, flagella are thread-like protoplasmic extensions used to propel flagellates. Flagella have the same basic structure as cilia but are longer in proportion to the cell bearing them and present in much smaller numbers.

fungi Major group of heterotrophic, eukaryotic, single-celled, multinucleated, or multicellular organisms, including yeasts, molds, and mushrooms.

genotype Genetic makeup, as distinguished from physical and biochemical traits, of an organism or a group of organisms. Genotype is a set of fixed genetic characteristics, while phenotype is the subset of expressed characteristics. (Contrast with phenotype.)

granite A common, coarse-grained, light-colored, hard igneous rock consisting chiefly of quartz, orthoclase or microcline, and mica, and occurring as massive geologic formations, especially in mountain ranges.

genome The complete set of DNA carried by an organism. In prokaryotes, this means chromosomal plus plasmid DNA.

heterotroph Organism requiring an organic form of carbon as a carbon source. Heterotrophs do not fix CO_2.

horizontal gene transfer Horizontal gene transfer is any process in which an organism transfers genetic material (i.e., DNA) to another cell that resides in another branch of the evolutionary tree (lines of descent).

humic substances A series of relatively high molecular weight, yellow- to black-colored organic substances formed by secondary synthesis reactions in soils. The term is used in a generic sense to describe the colored material or its fractions

obtained on the basis of solubility characteristics. These materials are distinctive to soil environments in that they are dissimilar to the biopolymers of microorganisms and higher plants (including lignin).

humus A brown or black organic substance consisting of partially or wholly decayed vegetable or animal matter that provides nutrients for plants and increases the ability of soil to retain water (*see* humic substances).

hypha, hyphae Tubular structures that constitute the basic cellular unit of most fungi.

hypoxia An environmental condition featuring reduced oxygen concentration.

igneous Geology. Resulting from, or produced by, the action of fire. Lavas and basalt are igneous rocks.

intrinsic bioremediation Elimination of pollutants at a given site by the naturally occurring microbial populations functioning in naturally occurring chemical, biological, and geologic conditions. Also known as natural attenuation when dominated by biological processes.

Koch's postulates A set of criteria, devised by Robert Koch in 1884, to establish causality between a pathogenic microorganism and its impact on a diseased host.

lateral gene transfer *See* horizontal gene transfer.

ligand An ion, a molecule, or a molecular group that binds to another chemical entity to form a larger complex.

lithotroph Physiology. Term used in reference to an organism's energy source (electron donor); an organism that uses an inorganic substrate (such as ammonia, hydrogen) as an electron donor in energy metabolism. Literally, lithothroph means "rock eater".

lysis To break open a cell.

lysogeny The condition of a host bacterium that has incorporated a virus into its own genetic material. When a virus infects a bacterium it can either destroy its host (lytic cycle) or be incorporated as a prophage in the host genome in a state of lysogeny.

metabolism The integration of cellular biochemical reactions; often subdivided into catabolism (generation of energy as ATP) and anabolism (biosynthetic reactions that build and maintain new cells at the expense of ATP).

metamorphic Geology. Pertaining to, produced by, or exhibiting certain changes that minerals or rocks may have undergone since their original deposition; especially applied to the recrystallization that sedimentary rocks have undergone through the influence of heat and pressure, after which they are called metamorphic rocks.

micron (μm) A unit of length equal to one-millionth (10^{-6}) of a meter.

mineralization Conversion of organic substances to inorganic ones. For example, the production of CO_2 to NO_3^- from decaying organic matter.

mRNA Messenger RNA, the key intermediary in gene expression. mRNA serves as template for translation of the genetic code into chains of amino acids that constitute proteins.

mycelium, mycelia Fungal biomass, a network of hyphae.

natural attenuation Generally refers to the physical, chemical, or biological processes that, under favorable conditions, lead to the reduction of mass, toxicity, mobility, volume, or concentration of organic contaminants in soil, sediment, and/or groundwater. The reduction takes place as a result of processes such as biological or chemical degradation, sorption, and others.

obligate The ability to live in only a set parameter of conditions; for example, an obligate anaerobe can survive only in the absence of oxygen.

oligocarbophile Able to grow only on highly diluted carbon substrates (*see* oligotroph).

oligotroph A physiological type of microorganism (or phase in the life of marine microorganisms) that is suited to growth on nutrients at low concentration.

oligotrophic (*adj.*) habitat state exhibiting very low mineral and organic nutrients.

organotroph Microbial nutrition dependent upon organic compounds.

orthologous (*adj.*) Genes that are related by descent; they have evolved directly from an ancestral gene.

osmotroph An organism that obtains nutrients through the active uptake of soluble materials across the cell membrane. This class of organism, which includes the prokaryotes and fungi, cannot directly utilize particulate material as nutrients. (Contrast with phagotroph.)

OTU Operational taxonomic unit; this often refers to distinctive 16S rRNA gene sequences retrieved from environmental samples. OTU is the safe terminology in environmental microbiology because it avoids the use of the term "species" (which are very difficult to define).

oxidative phosphorylation Use of electron transport to produce ATP, via proton motive force

oxidative stress A state of metabolic imbalance caused by increased levels of free radicals and other oxidation-promoting molecules that may result in cell membrane damage, cell death, and damage to genetic material.

PCR Polymerase chain reaction; a technique for amplifying DNA, making it easier to isolate, clone, and sequence.

paralogous (*adj.*) Two genes (or clusters of genes) at different chromosomal locations in the same organism that have structural similarities indicating that they have been derived from a common ancestral gene and have since diverged from the parent copy by mutation or selection or drift.

Petri dish A shallow glass or plastic cylindrical dish that microbiologists use to culture microorganisms. It was named after the German bacteriologist Julius Richard Petri (1852–1921) who invented it in 1877 when working as an assistant to Robert Koch.

phage Viruses that infect prokaryotes.

phagotroph An organism that engulfs is food. (Contrast with osmotroph.)

phenotype The observable physical or biochemical characteristics of an organism, as determined by both genetic makeup and environmental influences. (Contrast with genotype.)

photosynthesis A biochemical process in which plants, algae, and some prokaryotes harness the energy of light. The "light reactions" generate ATP and reducing power; the dark reactions fix CO_2 into biomass.

phylogeny Phylogeny documents the evolutionary relationship between organisms. The phylogeny of a particular organism reflects its ancestry, its own evolutionary developments.

phylum, phyla Used in classification and taxonomy. A phylum is a broad group of related microorganisms and contains one or more classes. A group of similar phyla forms a domain.

planetisemal Astronomy. Any of innumerable small bodies thought to have orbited the Sun during the formation of the planets.

plasmid Extrachromosomal DNA that replicates independently of the chromosome. The metabolic functions encoded by plasmid DNA typically are peripheral (e.g., catabolism of unusual carbon compounds), and not essential for cell function.

plates Microbiology. Plates refer to Petri dishes containing a microbial growth medium, usually solidified with agar.

prokaryote A unicellular organism lacking a nuclear membrane, a discrete nucleus, and other specialized compartments within the cell. *Bacteria* and *Archaea* are prokaryotes. The use of the term "prokaryote" is historical baggage from early microbiologists' inability to distinguish between *Bacteria* and *Archaea*. In contemporary biology, the term is inherently inaccurate and often deemed obsolete. Because *Bacteria* and *Archaea* are vastly different (phylogenetically and phenotypically), referring to these two domains with a single term may cause erroneous thought.

proton motive force Generated by electron transport, this is the separation of H^+ and OH^- ions across biological membranes; used by the ATP synthase enzyme to generate ATP.

quorum sensing The ability of populations of prokaryotes to communicate and coordinate behavior via signaling molecules.

16S rRNA and 18S rRNA The "16S" designation refers to "Svedberg units", which are sedimentation coefficients derived from the physical means of separating components of ribosomes from one another. Prokaryotes have 16S, while eukaryotes have 18S ribosomal RNA subunits. (*See* rRNA; small subunit rRNA.)

rDNA DNA sequences coding for rRNA.

rRNA Ribosomal RNA; major structural component of ribosomes. Ribosomes are the site of protein synthesis in cellular life. (*See* small subunit rRNA.)

redox Abbreviation for the coupling of "oxidation" (electron donating) and "reduction" (electron receiving) reactions.

regulatory gene A DNA sequence whose encoded protein (after transcription and translation) controls the expression of other genes. (Contrast with structural gene.)

resource A metabolic asset, present in the habitat of a microorganism, that can be used in biochemical reactions to generate ATP and/or be used as cellular building blocks to create new cells.

respiration Membrane-based coupling of electron flow (via electron carriers undergoing redox reactions) between a reduced energy source (organic or inorganic) and an oxidized terminal electron acceptor (e.g., oxygen or nitrate).

scanning electron microscopy (SEM) Any of a class of microscopic procedures that use electrons rather than visible light to produce magnified images, especially of objects having dimensions smaller than the wavelengths of visible light, with linear magnification approaching or exceeding a million (10^6).

sedimentary Rocks formed from material, including debris of organic origin, deposited as sediment by water, wind, or ice and then compressed and cemented together by pressure.

senescence Decline or degeneration of cellular function, as with maturation, age, or disease stress.

slime mold Any of various single-celled eukaryotes that grow on dung and decaying vegetation and have a life cycle characterized by a slime-like amoeboid stage and a multicellular reproductive stage.

small subunit rRNA Ribosomal RNA from the 30S ribosomal subunit of prokaryotes or the 40S ribosomal subunit of eukaryotes. Small subunit rRNA plays a

crucial cellular role in the translation of mRNA into protein. Used in molecular phylogeny to establish the three domains of life. (*See* rRNA.)

spore A small, usually single-celled, reproductive body that is highly resistant to desiccation and heat and is capable of growing into a new organism, produced especially by certain prokaryotes, fungi, algae, and nonflowering plants.

structural gene A DNA sequence whose encoded protein (after transcription and translation) has a definite catalytic or structural role in cell metabolism. (Contrast with regulatory gene.)

sulfatara A volcanic area or vent, characterized by high temperature, sulfur vapors, and steam.

taxonomy The science of identification, classification, and nomenclature.

thallus Fungal biomass, a network of mycelia.

tomography Any of several techniques for making detailed X-rays of a predetermined plane section of a solid object while blurring out the images of other planes. Multiple planar images can then be assembled.

transduction The transfer of microbial DNA by viruses from one infected microorganism to another.

transformation A process by which the genetic material carried by an individual microbial cell is altered by incorporation of exogenous DNA into its genome. The source DNA taken up by the recipient cell is free of any vector (no virus, no plasmid).

transmission electron microscopy (TEM) Procedure using a beam of highly energetic electrons to examine objects very closely, on a fine scale. The microscope shines a beam of electrons through an object and the transmitted result is projected on to a phosphor screen.

tree of life Graphic presentation of evolutionary relationships between past and present forms of life. The current dominant paradigm for evolution is based on phylogenetic analysis of genes encoding small subunit rRNA.

tRNA Short-chain RNA molecules present in the cell (in at least 20 varieties, each variety capable of combining with a specific amino acid) that attach the correct amino acid to the protein chain that is being synthesized by the ribosome of the cell.

ultraviolet light (UV) Light waves that have a shorter wavelength than visible light, but a longer wavelength than X rays.

unculturable (*adj*.) A misleading term that should never be used in microbiology in a taxonomic sense that classifies microorganisms. All microorganisms that have evolved on Earth have the potential to be cultured (domesticated) if and when the proper growth medium is devised. However, individual microbial cells may be damaged or moribund, and hence be unable to replicate and grow; under such circumstances their physiological state renders them "unculturable", unable to replicate.

vertical gene transfer Vertical gene transfer occurs when an organism receives genetic material from its ancestor, e.g., its parent or a species from which it evolved.

viable plate counts A way to count culturable microorganisms in environmental samples, on solid agar media. The number of microbial colonies that grow on agar plates is inversely proportional to the degree the environmental sample was diluted.

virus A small particle, containing DNA or RNA, that infects cells. Viruses are obligate intracellular parasites; they can reproduce only by invading and taking over other cells as they lack the cellular machinery for self-reproduction.

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